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13. ABSTRACT (Maximum 200 Words) The effects of androgens are mediated by the androgen receptor (AR), which plays a critical role in inducing normal differentiation of tissues of the reproductive organs and in the development and progression of prostate cancer. The cell cycle signaling regulated by the mitogen activated protein/extracellular-signal-regulated kinase (MAPK/ERK) have been linked to tumor development and progression. The p21-activated kinases (PAKs) are members of Rac/Cdc42-associated Ste20-like ser/thr protein kinases. Previous studies have shown that MAPK/ERK signaling can be mediated via Cdc42/Rac-stimulation of PAK activity. Our finding that AR interacts with a new PAK, PAK6, provided the first link between PAK signaling to the steroid hormone receptor pathway. In this study, we proposed two sets of experiments to further assess biological roles of PAK6 in prostate cancer cells, and to examine the expression of PAK6 in prostate tissues. We anticipate that by completing the above objectives, we will obtain fresh insight into understanding the regulatory processes of the interaction between AR and PAK6, which may contribute to the development of new targets for the treatment of prostate cancer.				
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INTRODUCTION:

Prostate cancer is the most common malignancy in men and the second leading cause of cancer deaths in the United States (Landis et al., 1999). The androgen signaling pathway, which is mainly mediated through the androgen receptor (AR), is important for the normal and neoplastic development of prostate cells (Balk, 2002; Gelmann, 2002). Androgen ablation is an effective treatment for the majority of advanced prostate cancer patients (Kyprianou and Isaacs, 1988). However, most of the patients develop androgen-insensitive prostate cancer within two years, for which there is currently no effective treatment. Multiple mechanisms by which prostate cancer cells progress to androgen-insensitive stages have been proposed (Balk, 2002; Jenster, 1999). Experiments with AR and other steroid receptors have identified several associated proteins that can modulate the receptor-mediated transcriptions (Rosenfeld and Glass, 2001). It has also been shown that growth factor stimulated signal transduction pathways can modulate the activity of AR through modification of the protein such as phosphorylation (Blok et al., 1996; Lin et al., 2001). A novel AR-interacting protein, PAK6, was identified in our group previously (Yang et al., 2001). PAK6 is a 75 kDa protein that contains a putative amino-terminal Cdc42/Rac Interactive Binding (CRIB) motif and a carboxy-terminal kinase domain. The goal of this study is to determine the biological roles of PAK6 in the tumorigenesis of prostate cancer. Two specific aims, including assessing the role of PAK6 in cell proliferation and detecting alterations in PAK6 expression in prostate cancer tissues, have been proposed and approved by both the scientific and programmatic reviewers. We have made significant progress within this funding period, which are summarized followed.

BODY:

Multiple lines of evidence have shown that the majority of primary prostate cancers are dependent upon the AR and blocking the AR is an extremely important method for treating prostate cancer (Balk, 2002; Gelmann, 2002). The p21-activated kinases (PAKs) are members of a growing class of Rac/Cdc42-associated Ste20-like ser/thr protein kinases, characterized by a highly conserved amino-terminal Cdc42/Rac Interactive Binding (CRIB) domain and a carboxy-terminal kinase domain (Abo et al., 1998; Bagrodia et al., 1998; Knaus et al., 1995; Manser et al., 1994; Martin et al., 1995). PAK proteins are the direct effectors of the Rho family of GTPases, Rac1 and Cdc42. The GTPases bind to a conserved p21-binding domain and stimulate their serine/threonine kinase activities (Hoffman and Cerione, 2000; Knaus and Bokoch, 1998). Although the biological functions of PAKs remain unclear, PAKs are implicated in the regulation of a number of cellular processes, including rearrangement of the cytoskeleton, apoptosis, mitogen-activated protein kinase (MAPK) signaling pathway, growth factor-induced neurite outgrowth, and control of phagocyte NADPH oxidase (Bokoch et al., 1998; Burbelo et al., 1995; Knaus and Bokoch, 1998; Widmann et al., 1998; Zigmond et al., 1997). Interactions between the AR and PAK6 pathways may be an important mechanism both for the development and progression of prostate cancer. In our original grant application, we proposed four specific tasks to investigate the biological roles of PAK6 and the significance of its interaction with AR, including (1) To characterize the biological roles of PAK6 in prostate cancer cells, (2) To detect expression of PAK6 protein in prostate cancer samples by immunohistochemistry, (3) To elucidate the interaction between PAK6 and AR in prostate cancer cells, and (4) To study the mechanisms by which PAK6 represses AR-mediated transcription. However, both the scientific

and programmatic reviewers recommended only funding tasks 1 and 2. To reflect these changes, we have revised our specific aims when the project started. Some of our data has been published recently (Schrantz et al., 2004)

Objective 1: To characterize the biological roles of PAK6 in prostate cancer cells.

The sequence and structure of PAK6 suggest that the protein may possess the biological functions comparable to other PAK members. The selective expression of PAK6 in testis and prostate tissues is consistent with the interaction between AR and PAK6 that was identified in our previous study (Yang et al., 2001). PAK6 was also identified to bind to AR and to inhibit AR mediated transcription. We have further study biological roles of PAK6 in androgen signaling. We first investigated the role of the kinase activity of PAK6 in this inhibition. We compared the kinase activity of PAK6 with two other members of the PAK family PAK1 and PAK4. Like PAK4, PAK6 possesses a constitutively kinase activity. However, the kinase activity of PAK6 is not modulated by GTPases, such as Rac/Cdc42, which is different from PAK1. To study the involvement of PAK6 kinase activity in AR-mediated transcription, we generated the kinase dead (KD) and kinase active (KA) mutants of PAK6. Interestingly, transient transfection experiments showed that PAK6 kinase activity is implicated in PAK6 repressed AR-mediated transcription. The wild type (WT) form of PAK6 showed a strong inhibitory effect on AR-mediated transcription as we observed previously (Yang et al., 2001), while the KD and the KA mutants of PAK6 inhibited AR activity by 20% and 90%, respectively. Importantly, either kinase dead or constitutively active mutants of PAK1 and PAK4 did not show a significant effect on AR-mediated transcription. These results demonstrated that PAK6 repression of AR-mediated transcription is dependent on the kinase activity of PAK6.

One possible mechanism for repression by PAK6 could be due to disrupting recruitment of other co-activators to the AR-complex. Several AR co-activators that specifically interact with AR and enhance AR transactivation have been identified (Hsiao and Chang, 1999). Co-transfection of AR and cofactor including β -catenin, SRC1 and ARA55 showed augmentations of AR-mediated transactivation in PC3 and LNCaP cells. In the presence of PAK6 WT and PAK6 KA, enhancements of AR activity by these cofactors were significantly reduced. Although it is not clear how PAK6 affects the cofactors in augmenting AR activity, our data suggests that the inhibition of AR and cofactors by PAK6 may be mediated through modification of the phosphorylation of these proteins. Therefore, we directly examined whether PAK6 affects the phosphorylation status of AR. Expression vectors of PAK6 WT, KA, and KD were co-transfected with different AR truncated mutants into CV1 cells. The transfected cells were incubated with medium with 32 P-orthophosphate in the presence or absence of the appropriate ligands. After 12-24 hrs, different fragments of AR proteins were immunoprecipitated with the Flag antibody from the CV1 cells and analyzed on SDS-PAGE. Through these analyses, we demonstrated that PAK6 WT and KA were able to phosphorylate the DBD domain of AR.

To better understand the potential biological role(s) of PAK6, we examined the expression of the PAK6 protein in different human cancer cell lines. The antiphospho-PAK6 (Ser(P)-560) antibody was used to test both prostate cancer cell lines, DU145, PC-3, ARCaP, LNCaP, and LAPC4, and non-prostate cancer cell lines, MCF7 and HeLa. The western-blot showed that phospho-PAK6 was detected most abundantly in the LAPC4 cell line. High expression/activity

levels were also detected in the DU145 and PC-3. MCF7 and ARCaP expressed only a small amount of phospho-PAK6, and no expression was detected in HeLa and LNCaP. These results are consistent with our Northern blot results (Yang et al., 2001) and indicate that the expression and/or activity level of PAK6 in prostate cell lines is highly variable, suggesting that PAK6 could be involved in differential steps of AR signaling in these cells.

To further study the biological role of PAK6 in the tumorigenesis of prostate cancer, we made several sublines of LNCaP cells that were stably transfected with wt and mut of PAK6 constructs. We confirmed the expression of PAK6 in these cells and have assessed their growth rates by examining cell proliferation, colony formation, and cell cycle progression. We also generated the PAK6 adenoviral siRNA constructs. With these tool reagents, we will be able to continue investigating the biological role of PAK6 and the interaction between AR and PAK6 in the tumorigenesis of prostate cancer.

Objective 2: To detect expression of PAK6 protein in prostate cancer samples by immunohistochemistry.

During this funding period, we have spent significant effort to search for a direct link between PAK6 and the tumorigenesis of prostate cancer by assessing the expression of the PAK6 protein in human prostate cancer tissues. We attempted to determine whether the expression of the PAK6 is altered at different stages of prostate cancers. Particularly, we are very interested in whether this alteration occurs in the late stage of prostate cancer, and therefore, whether we can use PAK6 as a marker for predicting prostate cancer progression.

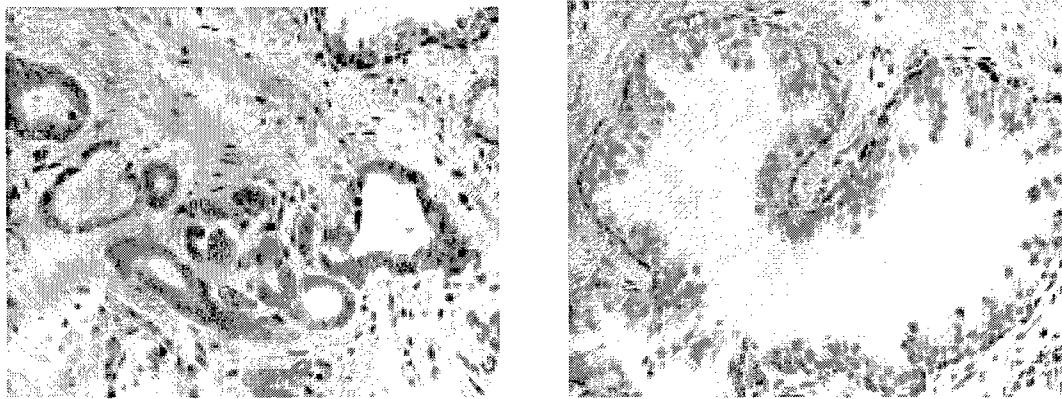


Figure 1: Immunohistochemistry of human prostate cancer tissues with the anti-PAK6 antibody.
Two prostate tissue samples were stained with the rabbit polyclonal antibody against human PAK6 protein. Color was developed with the ImmunCruz Staining System (Santa Cruz, sc-2023). Sections were lightly counterstained with 5% (w/v) hematoxylin.

Using the homemade rabbit polyclonal antibody against human PAK6, we examined the expression of the PAK6 in human prostate tissues from the prostate tissue bank in the Department of Urology at Stanford Medical Center. So far, 10 samples from normal, BPH, and tumor tissues have been examined (see Figure 1). In our experiments, we used the standard

three-step immunoperoxidase-based method as described previously (McNeal et al., 1995). The PAK6 antibody, at a 1:100 dilution, was added to the slides and incubated overnight at 4°C. After three washes, slides were incubated with biotinylated secondary antibody and washed again. Color was developed with the ImmunoCruz staining system (Santa Cruz, sc 2053). Interestingly, we did not observe strong staining in tissues, and instead, only a few of cells that seem to be stained with the antibody in the luminal epithelial areas.

Although the above anti-PAK6 antibody has been successfully used in some of our experiments to detect the over-expressed protein, it seems that it did not work in our tumor samples. For this reason, we have made significant effort to generate new PAK6 antibodies. So far, four different antibodies have been produced and we are in the process of assessing their sensitivities and specificities. We hope that these new antibodies will be useful for our future studies. In addition, we recently have started *in situ* hybridization in collaborating with the Pathology Department at Stanford Medical Center. Our efforts will be adjusted based on our progresses in the future.

KEY RESEARCH ACCOMPLISHMENTS:

- 1) Demonstrate that PAK6-induced inhibition of AR-mediated transcription is independent of Cdc42 binding.
- 2) Identify that the kinase domain of PAK6 is involved in repression of AR activity.
- 3) Demonstrate that PAK6 phosphorylates the DBD of AR.
- 4) Construct PAK6 SiRNA adenoviral vectors and adenoviral vectors of PAK6.
- 5) Establish LNCaP sublines that are stably transfected with wild type and mutants of PAK6.

REPORTABLE OUTCOMES:

Publications:

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2. Sharma M, Chuang WW, Sun ZJ. (2002). Regulation of androgen signaling by the phosphatidylinositol 3-kinase/Akt is mediated through GSK3beta/beta-catenin pathway. *J. Biol. Chem.* 277:30935-30941.
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Abstracts:

N. M. Schrantz, Z. Sun & G. M. Bokoch. Repression of AR-mediated transcription by PAK6 is dependent on PAK6 kinase activity. ASCB, San Francisco, Dec, 2002.

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Beta-catenin and androgen signaling in prostate cancer
3. 2003-2006 The Dept. of Defense/PC020763, Principal Investigator: Zijie Sun
PTEN regulates beta-catenin in androgen signaling: Implication in prostate cancer progression

CONCLUSIONS:

In this funding period (2002-2005), we have further investigated the molecular mechanism(s) by which PAK6 regulates AR-mediated transcription. We demonstrated that PAK6-induced inhibition of AR-mediated transcription is independent of Cdc42 binding and dominant over the presence of the AR co-activators. The expression of PAK6 protein in different cancer cell lines was also assessed. In addition, we also examined the expression of the PAK6 protein in human prostate cancer tissues. These data will provide fresh insight into understanding the biological role of PAK6 in the tumorigenesis of prostate cancer.

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Linking β -Catenin to Androgen-signaling Pathway*

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The androgen-signaling pathway is important for the growth and progression of prostate cancer cells. The growth-promoting effects of androgen on prostate cells are mediated mostly through the androgen receptor (AR). There is increasing evidence that transcription activation by AR is mediated through interaction with other cofactors. β -Catenin plays a critical role in embryonic development and tumorigenesis through its effects on E-cadherin-mediated cell adhesion and Wnt-dependent signal transduction. Here, we demonstrate that a specific protein-protein interaction occurs between β -catenin and AR. Unlike the steroid hormone receptor coactivator 1 (SRC1), β -catenin showed a strong interaction with AR but not with other steroid hormone receptors such as estrogen receptor α , progesterone receptor β , and glucocorticoid receptor. The ligand binding domain of AR and the NH₂ terminus combined with the first six armadillo repeats of β -catenin were shown to be necessary for the interaction. Through this specific interaction, β -catenin augments the ligand-dependent activity of AR in prostate cancer cells. Moreover, expression of E-cadherin in E-cadherin-negative prostate cancer cells results in redistribution of the cytoplasmic β -catenin to the cell membrane and reduction of AR-mediated transcription. These data suggest that loss of E-cadherin can elevate the cellular levels of β -catenin in prostate cancer cells, which may directly contribute to invasiveness and a more malignant tumor phenotype by augmenting AR activity during prostate cancer progression.

Prostate cancer is the most commonly diagnosed malignancy among males in western countries (1). However, in contrast to some other tumors, the molecular events involved in the development and progression of prostate cancer remain largely unknown. Androgen ablation, used as an effective treatment for the majority of advanced prostate cancers, indicates that androgen plays an essential role in regulating the growth of prostate cancer cells. The growth-promoting effects of androgen in prostate cells are mediated mostly through the androgen receptor (AR).¹ There is increasing evidence that the nuclear

hormone receptors, including AR, interact with other signal transduction pathways (2). The regulation by cofactors can modulate AR activities, which may contribute to the development and progression of prostate cancer.

β -Catenin plays a pivotal role in cadherin-based cell adhesion and in the Wnt-signaling pathway (3, 4). Corresponding to its dual functions in the cells, β -catenin is localized to two cellular pools. Most of the β -catenin is located in the cell membrane where it is associated with the cytoplasmic region of E-cadherin, a transmembrane protein involved in homotypic cell-cell contacts (5). A smaller pool of β -catenin is located in the nucleus and cytoplasm and mediates Wnt signaling. In the absence of a Wnt signal, β -catenin is constitutively down-regulated by a multicomponent destruction complex containing GSK3 β , axin, and a tumor suppressor, adenomatous polyposis coli (APC). These proteins promote the phosphorylation of serine and threonine residues in the NH₂-terminal region of β -catenin and thereby target it for degradation by the ubiquitin proteasome pathway (6). Wnt signaling inhibits this process, which leads to an accumulation of β -catenin in the nucleus and promotes the formation of transcriptionally active complexes with members of the Tcf/LEF family (7). Activation of Tcf/LEF and β -catenin targets has been shown to induce neoplastic transformation in cells, suggesting a potential role of β -catenin in tumorigenesis (8).

The link between stabilized β -catenin and tumor development and progression was considerably strengthened by discoveries of mutations in both β -catenin and components of the destruction complex in a wide variety of human cancers, which cause increased cellular levels of β -catenin (3, 9). About 85% of all sporadic and hereditary colorectal tumors show loss of APC function, which correlates with the increased levels of free β -catenin found in these cancer cells (10–12). It appears that inappropriate high cellular levels of β -catenin play a fundamentally important role in tumorigenesis.

In normal epithelial tissues, E-cadherin complexes with actin cytoskeleton via cytoplasmic catenins to maintain the functional characteristics of epithelia. Disruption of this complex, due primarily to the loss or decreased expression of E-cadherin, is frequently observed in many advanced, poorly differentiated carcinomas (13, 14). There is a strong correlation between decreased expression of E-cadherin and an invasive and metastatic phenotype of human prostate cancers

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¹ The abbreviations used are: AR, androgen receptor; GR, glucocorti-

coid receptor; ER α , estrogen receptor α ; PR β , progesterone receptor β ; VDR, vitamin D receptor; TAD, transcription activation domain; DBD, DNA binding domain; LBD, ligand binding domain; DHT, dihydrotestosterone; PSA, prostate specific antigen; ARE, androgen responsive element; MMTV, mouse mammary tumor virus; GST, glutathione S-transferase; ARA70, androgen receptor-associated protein 70; β -gal, β -galactosidase; APC, adenomatous polyposis coli.

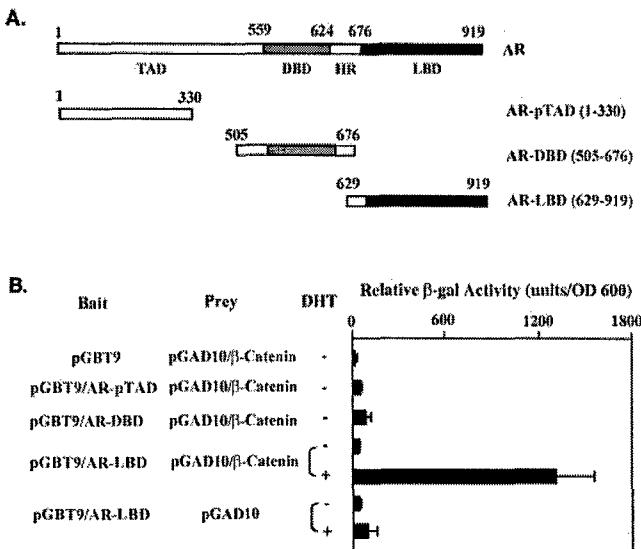


FIG. 1. Specific interaction of β -catenin with the LBD of AR. **A**, schematic representation of different portions of the human AR that were used in the yeast experiments. Numbers correspond to amino acid residues. **B**, full-length β -catenin clone or an empty library vector (pGAD10) was cotransformed into yeast strain PJ69 with either the bait vector (pGBT9), AR-pTAD, AR-DBD, or AR-LBD. Transformed cells were plated on SD-Ade-Leu-Trp plates with or without 100 nM DHT and SD-Leu-Trp plates for monitoring the transformation efficiency. Three independent colonies were inoculated from each transformation experiment for a β -gal assay. The data for the liquid β -gal assay is shown as the mean \pm S.D.

(15). Besides playing a role in retaining normal cell-cell contact, E-cadherin can also modulate the cytoplasmic pools of β -catenin for signaling (16).

Here, we demonstrated a specific protein-protein interaction between β -catenin and AR. Importantly, unlike the steroid receptor cofactor 1 (SRC1), β -catenin selectively binds to AR in a ligand-dependent manner but not to other steroid hormone receptors such as the estrogen receptor α (ER α), the progesterone receptor β (PR β), and glucocorticoid receptor (GR). The ligand binding domain (LBD) of AR and the central region spanning the armadillo repeats 1–6 of β -catenin were found to be responsible for the interaction. Using transient transfection experiments, we further demonstrated that β -catenin augments the ligand-dependent activity of AR in prostate cancer cells through this specific interaction. These data identify a new role for β -catenin in nuclear hormone receptor-mediated transcription. Moreover, transfection of an E-cadherin expression construct into an E-cadherin-negative prostate cancer cell line, TSU.pr-1, resulted in redistribution of β -catenin to the cell membrane and reduction of AR-dependent transcriptional activity. They suggest that reduced expression of E-cadherin can elevate the cellular levels of β -catenin in prostate cancer cells, which may directly contribute to the invasiveness and more malignant tumor phenotype by augmenting AR activity during the progression of prostate cancer.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid System—Yeast two-hybrid experiments were basically performed as described previously (17). The LBD of human AR (amino acids 629–919) was fused in frame to the GAL4 DBD in the pGBT9 vector (CLONTECH, Palo Alto, CA). The construct was transformed into a modified yeast strain PJ69-4A (18). A cDNA library from human brain tissue was used in this screening (CLONTECH). Transformants were selected on Sabouraud dextrose medium lacking adenine, leucine, and tryptophan in the presence of 100 nM dihydrotestosterone (DHT). The specificity of interaction with AR was determined by a liquid β -galactosidase (β -gal) assay as described previously (17). β -Gal activities were measured using the Galacto-light Plus kit (Tropix Inc.,

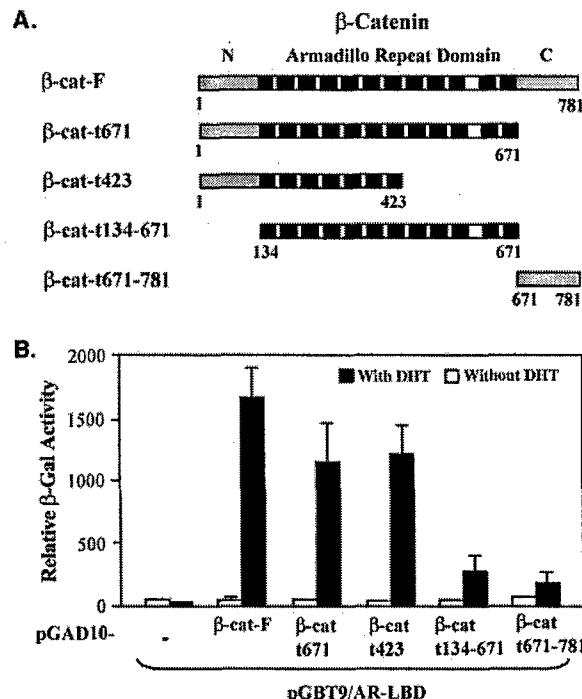


FIG. 2. Delineation of the domain in β -catenin that mediates the interaction with AR-LBD in the yeast two-hybrid system. **A**, both the NH₂- and COOH-terminal regions and a central armadillo domain of β -catenin are shown schematically. Numbers correspond to amino acid residues. Full-length (*F*) and truncated (*t*) β -catenin constructs were generated. **B**, the various truncated β -catenin genes in pGAD10 were cotransformed into yeast strain PJ69 with AR-LBD. Specific interactions between the two fusion proteins were measured by the appearance of colonies on SD-Ade-Leu-Trp plates and a liquid β -gal assay in the presence or absence of 100 nM DHT. A liquid β -gal assay is presented as the mean \pm S.D. of three independent colonies.

Bedford, MA) and normalized by cell density (A_{600}). pGBT9 constructs with three different AR fragments, including the partial TAD (amino acids 1–330), DBD (amino acids 505–676), and LBD were used to confirm the interaction.

Plasmid Construction—A yeast clone containing the full-length cDNA of human β -catenin was isolated in the screen. Using it as a template, the COOH-terminal and internal deletions of β -catenin clones were generated by PCR with specific primers containing the appropriate restriction enzyme sites. After cleavage, the fragments containing different portions of the β -catenin were cloned downstream of GAL4 TAD in the pGAD10 vector (CLONTECH). The LBD fragments of ER α (amino acids 250–602), PR β (amino acids 633–952), VDR (amino acids 90–427) were generated by PCR with specific primers and subcloned in-frame to the GAL4 DBD in pGBT9. An antisense construct of β -catenin containing the NH₂-terminal 513 bp was generated by PCR and cloned into the pcDNA3 vector at EcoRI site. All constructs were sequenced to confirm that there were no mutations introduced by PCR.

The AR expression vector, pSV-hAR, was provided by Dr. Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands). The expression constructs for human ER α and pERE-luc plasmid were generously given by Dr. Myles Brown (Dana-Farber Cancer Institute, Boston, MA). A human PR β and PRE-luc reporter were provided by Dr. Kathryn B. Horwitz (University of Colorado). The expression constructs of human GR and VDR, and the pVDRE-luc reporter plasmid, were the kind gifts of Dr. David Feldman (Stanford University, Stanford, CA). pSV- β -gal, an SV40-driven β -galactosidase reporter plasmid (Promega, Madison, WI) was used in this study as an internal control. The pSG5-ARA70 plasmid and the reporter plasmid pARE-luc were the kind gifts of Dr. Chawnshang Chang (19). pMMTV-pA3-luc was provided by Dr. Richard Pestell (Albert Einstein College of Medicine, New York). The reporter plasmids, pPSA7kb-luc, with the luciferase gene under the control of promoter fragments of the human prostate-specific antigen was obtained from Dr. Jan Trapman (20).

Cell Cultures and Transfections—The monkey kidney cell line, CV-1, was maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (HyClone, Denver, CO). An AR-positive pros-

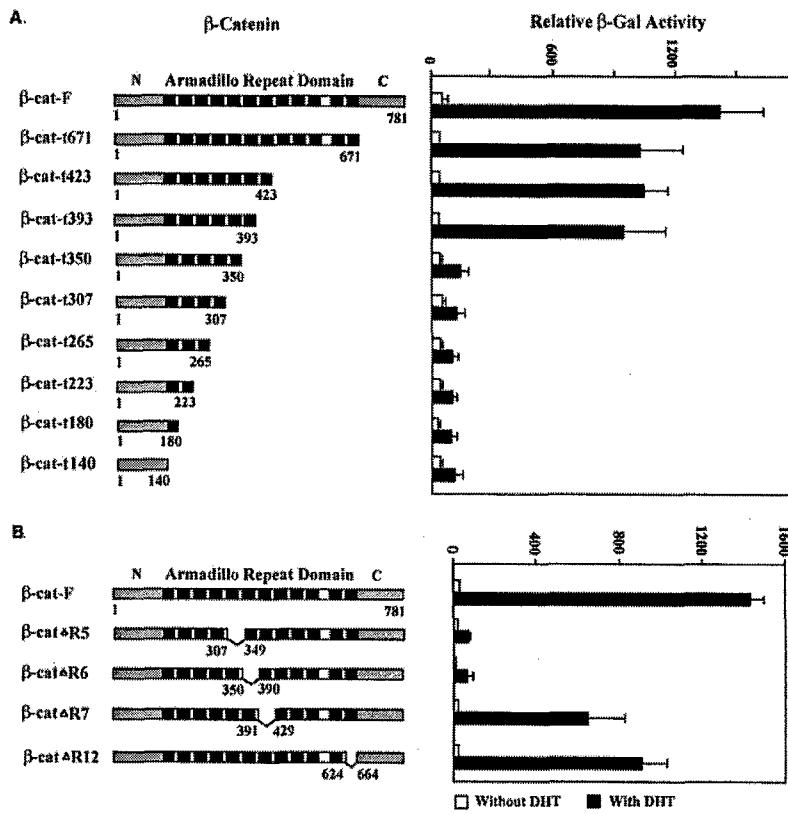


FIG. 3. Mapping the armadillo repeats in β-catenin that mediate the interaction with AR-LBD in yeast two-hybrid system. Mutants of β-catenin containing COOH-terminal truncations (A) and internal deletions (B) in pGAD10 were made and cotransformed into yeast strain PJ69 with pGBT9/AR-LBD in the presence or absence of 100 nM DHT. Numbers corresponding to amino acid residues are indicated for each construct. Liquid β-gal was measured as described in the legend to Fig. 2.

tate cancer cell line, LNCaP, was maintained in T-medium (Invitrogen) with 5% fetal calf serum. The two sublines derived from TSU.pr-1 prostate cancer cells (16) were maintained in RPMI 1640 medium with 10% fetal calf serum and G418 (500 µg/ml).

Transient transfections were carried out using a LipofectAMINE transfection kit (Invitrogen) for CV1 and LipofectAMINE 2000 (Invitrogen) for TSU.pr-1 and LNCaP cells. Approximately 1.5–2 × 10⁴ cells were plated in a 48-well plate 16 h before transfection. 12–16 h after transfection, the cells were washed and fed medium containing 5% charcoal-stripped fetal calf serum (HyClone) in the presence or absence of steroid hormones. Cells were incubated for another 24 h, and luciferase activity was measured as relative light units (21). The relative light units from individual transfections were normalized by β-galactosidase activity in the same samples. Individual transfection experiments were done in triplicate and the results are reported as mean relative light units/β-galactosidase (+S.D.).

In Vitro Binding Assay—GST-β-catenin fusion proteins were constructed in the pGEX-4T-1 vector (Amersham Biosciences, Inc.). Expression and purification of GST fusion proteins were performed according to the manufacturer's instructions. Full-length human AR proteins were generated and ³⁵S-labeled *in vitro* by the TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Equal amounts of GST fusion proteins coupled to glutathione-Sepharose beads were incubated with ³⁵S-labeled proteins at 4 °C for 2 h in the lysis buffer as described above. Beads were carefully washed three times with washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40). GST fusion proteins were then eluted by incubating with buffer containing 10 mM glutathione and 50 mM Tris-HCl, pH 8.0, for 10 min at room temperature. The bound proteins were analyzed by SDS-PAGE followed by autoradiography.

Immunofluorescence—CV-1 cells were plated onto gelatin-coated (2%) coverslips the day before transfection. The pcDNA3-AR and the wild type or mutants of β-catenin plasmids were cotransfected into cells with the LipofectAMINE-PLUS reagent (Invitrogen). After 2 h, transfected cells were fed with fresh medium plus/minus 10 nM DHT, incubated for 4 h, and then fixed for 10 min with 3% paraformaldehyde in phosphate-buffered saline and washed with 0.1% Nonidet P-40/phosphate-buffered saline buffer. Nonspecific sites were blocked with 5% skim milk powder in phosphate-buffered saline for 30 min. The cells were then incubated with either anti-FLAG monoclonal or anti-AR polyclonal antibody for 1 h at room temperature. Cells were washed three times followed by incubation with fluorescein isothiocyanate-conjugated anti-mouse or

rhodamine-conjugated anti-rabbit secondary antibody (Santa Cruz Biotechnology). Indirect immunofluorescence staining was performed according to the procedure described previously (16). In TSU cells, E-cadherin was stained with the rat monoclonal antibody against uvomorulin (6 µg/ml; Sigma) and donkey anti-rat immunoglobulin conjugated to Alexa-488 (20 µg/ml; Molecular Probes, Eugene, OR). β-Catenin was stained with a mouse monoclonal anti-β-catenin antibody conjugated to TRITC (10 µg/ml; Transduction Laboratories).

RESULTS

Androgen Receptor Interacts with β-Catenin in a Ligand-dependent Manner—Using a bait construct containing the LBD and hinge region of the AR, we employed a modified yeast two-hybrid system to identify proteins that interact with AR in an androgen-dependent manner (17, 18). Of 2 × 10⁷ transformants, 73 grew under selective conditions and showed increased adenine and β-gal production in medium containing 100 nM DHT. Rescue of the plasmids and sequencing of the inserts revealed several different cDNAs, including the previously identified SRC1 (22), an AR-associated protein (ARA70) (19), and several other AR-interacting proteins identified recently by others or us (17, 21, 23). Importantly, 23 of these clones perfectly matched the sequence of the full-length coding region of β-catenin. To confirm the interaction, we cotransformed one of these β-catenin clones with various constructs containing either GAL4DBD alone or the AR fusion proteins with a partial transactivation domain (pTAD), the DBD, and the LBD (Fig. 1A). pGAD10-β-catenin showed a specific interaction with GAL4DBD-AR-LBD by producing adenine in the presence of 100 nM DHT (data not shown). In the liquid β-gal assays, pGAD10-β-catenin showed an ~97-fold induction with pGBT9-AR-LBD in the presence of DHT (Fig. 1B). This result demonstrated that the LBD of AR specifically interacts with β-catenin in a ligand-dependent manner.

Armadillo Domain of β-Catenin Is Responsible for Binding to AR-β-Catenin and its Drosophila homolog, armadillo, contain a central core domain of 12 armadillo repeats flanked by

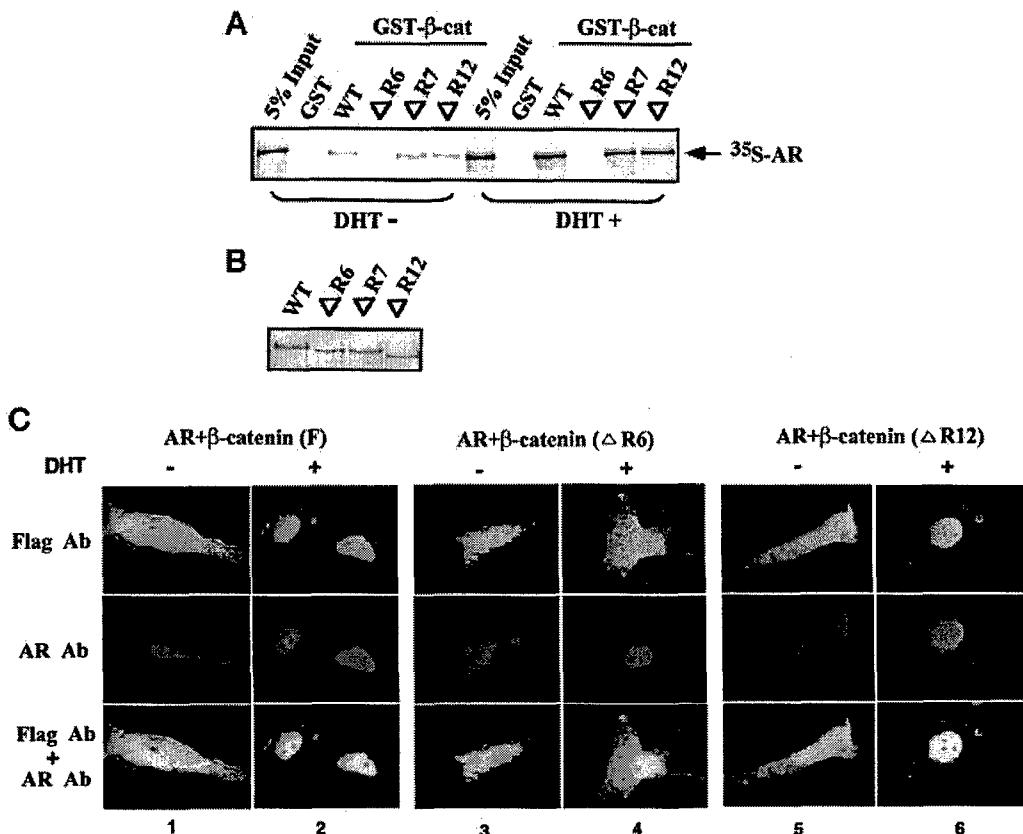


FIG. 4. β -Catenin interacts with AR *in vitro* and *in vivo*. *A*, GST fusion proteins containing full-length (WT), and different internal deletion mutants of β -catenin as indicated in the legend to Fig. 3*B* were constructed. The conjugated GST fusion proteins were incubated with *in vitro* expressed and ^{35}S -labeled full-length AR in the presence or absence of 10 nM DHT for 2 h at 4 °C and then eluted by 10 mM GSH in 50 mM Tris-HCl, pH 8.0, and resolved by 10% SDS-PAGE. *B*, GST fusion proteins were resolved in SDS-PAGE and stained with Coomassie Blue for measuring expression. *C*, CV-1 cells were transfected with an AR expression vector and FLAG-tagged wild type or mutants of β -catenin plasmids as indicated in the figure. The cells were cultured in the presence or absence of 10 nM DHT. AR proteins were detected with a polyclonal AR antibody and revealed by rhodamine-conjugated secondary antibody (red). FLAG-tagged β -catenin proteins were detected with a monoclonal anti-FLAG antibody and revealed with fluorescein isothiocyanate-conjugated secondary antibody (green).

unique NH₂ and COOH termini (7). To identify the region of β -catenin that interacts with AR, we generated several truncated mutants of β -catenin and assessed their ability to interact with AR using the yeast two-hybrid system (Fig. 2*A*). As shown in Fig. 2*B*, deletion of the COOH-terminal activation domain of β -catenin (β -cat-t671) alone, or in combination with the last five armadillo repeats (β -cat-t423), did not significantly affect the binding. However, a mutant in which the NH₂-terminal activation domain alone (β -cat-t134–671), or in combination with the central armadillo domain (β -cat-t671–781) was deleted, showed no interaction. This result suggests that the primary binding region for AR spans the NH₂ terminus and the first seven armadillo repeats of β -catenin.

To precisely map the interacting region, a series of truncated mutants were made in which each single armadillo repeat was subsequently deleted (Fig. 3*A*). The deletion constructs containing the NH₂-terminal region and the first six repeats (β -cat-t393) showed about two-thirds the activity of the full-length protein (β -cat-F) (Fig. 3*A*). However by deleting repeat 6 (β -cat-t350), the interaction was essentially abolished, indicating that armadillo repeat 6 is crucial for binding to AR. Deletion of repeats 1–5 obviously had little further effect.

It has been shown that most β -catenin-binding proteins such as Tcf/LEF family members (24), axin (25), APC (26, 27), and cadherins (26, 28) bind β -catenin mainly through the central armadillo repeats. In most of cases, the first 10 repeats are required for the interactions, and a minimum of 6–7 repeats are sufficient for detectable binding (29, 30). Those data are

consistent with our finding that deletion of repeat 6 fully abolished the interaction with AR. To more precisely map the interaction region within the first six armadillo repeats, we used a PCR-based, site-directed mutagenesis techniques to generate several internal deletion mutants. As shown in the figure, the wild type β -catenin and the mutant with deletion of repeat 7 ($\Delta R7$) or 12 ($\Delta R12$) all reacted avidly with the AR-LBD. In contrast, mutants lacking repeat 6 ($\Delta R6$) showed no interaction with the AR-LBD (Fig. 3*B*). Moreover, deletion of repeat 5 alone also fully abolished the interaction, indicating that the armadillo repeats 1–5 may be also involved in the interaction. To confirm this result, an additional internal deletion mutant lacking repeat 3 ($\Delta R3$) was generated and tested. As we expected, the mutant also showed no interaction with AR (data not shown). Taken together, the results allow us to conclude that the region spanning armadillo repeats 1–6 is mainly responsible for binding to AR.

β -Catenin Interacts with AR in Vitro and in Vivo—Physical interaction between AR and β -catenin was further assessed by GST pull-down experiments. A series of GST fusion proteins with the full-length β -catenin and internal deletion mutants were generated and immobilized onto a glutathione-Sepharose matrix. The binding of [^{35}S]methionine-labeled AR protein to GST- β -catenin fusion proteins was analyzed by SDS-PAGE and detected by autoradiography. As shown in Fig. 4*A*, AR protein bound to the GST fusion protein containing wild type β -catenin and its mutants lacking either repeat 7 or 12. The interaction is more pronounced in the presence of DHT than in

the absence of DHT, and as much as 5% of the input protein was recovered (Fig. 4A). However, a significant reduction of binding was observed between the AR protein and the β -catenin mutants lacking repeat 6 when equal amounts of the GST fusion proteins were used in the experiments (Fig. 4B). These results are consistent with our observations from the yeast two-hybrid system and show a domain-dependent interaction *in vitro*.

To confirm that endogenous AR and β -catenin are physically associated in intact cells, coimmunoprecipitation assays were carried out to detect a possible protein complex in a prostate cancer cell line, LNCaP. Using specific antibodies, we further confirmed that AR and β -catenin proteins form a protein complex in LNCaP cells and the formation of AR and β -catenin complexes in these cells was also enhanced by DHT (data not shown). These results are consistent with a recent report by Truica and colleagues (31).

Next, we examined whether a dynamic interaction between β -catenin and AR existed in cells. FLAG-tagged vectors containing either full-length or mutants of β -catenin were transfected into CV-1 cells, and the expressed protein showed a cytoplasmic and nuclear distribution, which was not altered by treatment with DHT (data not shown). Overexpressed β -catenin protein with AR vector, in the absence of DHT, showed the same cellular distribution as transfection of β -catenin plasmid alone, while transfected AR protein is localized mainly in the cytoplasm (Fig. 4C, panels 1, 3, and 5). In the presence of DHT, AR proteins are fully translocated into the nuclei (panels 2, 4, and 6). Importantly, both the wild type (panel 2) and the $\Delta R12$ mutant of β -catenin (panel 6) showed increased levels of nuclear translocation when cotransfected with AR compared with cells transfected with the $\Delta R6$ mutant of β -catenin in which cytoplasmic staining of β -catenin persisted (panel 4). These results provide the first evidence that β -catenin can translocate into the nucleus as part of a complex with AR by an interaction through armadillo repeat 6.

β-Catenin Binds Selectively to the AR—To assess the possibility that β -catenin functions as a general coactivator of nuclear receptors, we examined the interaction of β -catenin with other members of the nuclear receptor family in yeast. The LBD of ER α , PR β , and VDR were generated and fused to GAL-DBD in the pGBT9 vector. These plasmids were cotransformed with either pGAD10- β -catenin or pGAD10-SRC1 as a positive control in the presence of corresponding ligands. The yeast transformants were grown on the selective media, and a liquid β -gal assay was performed to quantify the interactions. All receptors were shown to have a ligand-dependent interaction with the SRC1 (Fig. 5A), which is consistent with the previous reports (22, 32). However, β -catenin showed a strong interaction with AR but not with ER α and PR β . VDR showed a weaker interaction with β -catenin in comparison to SRC1. These results indicate that β -catenin selectively interacts with AR.

The specificity of interaction between β -catenin and AR proteins was further tested in CV1 cells. Since AR, GR, and PR β all can activate the MMTV promoter, we examined whether β -catenin is able to enhance GR and PR β activity under identical experimental condition. Transfection experiments were repeated with β -catenin and AR, GR, and PR β expression plasmids, along with a luciferase reporter plasmid regulated by an MMTV promoter containing the steroid hormone-responsive elements (33, 34). As shown in Fig. 5B, all receptors showed a ligand-dependent transactivation with the MMTV promoter. However, β -catenin specifically augmented only AR-mediated transcription but not GR and PR β (Fig. 5B). Taken together, our results suggest that β -catenin differs from SRC1 and selectively affects AR.

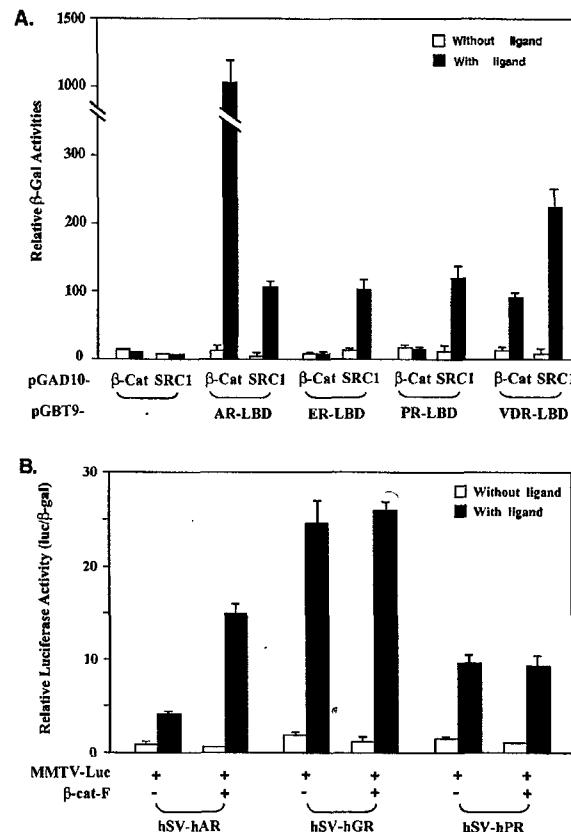


FIG. 5. β -Catenin specifically interacts with AR. *A*, the full-length β -catenin or SRC-1 in pGAD10 was cotransformed into yeast strain PJ69 with either the bait vector (pGBT9) or the fusion proteins containing the LBD of AR, ER α , PR β , or VDR. Transformed cells were plated on SD-Ade-Leu-Trp plates with or without 100 nM of DHT, 17 β -estradiol (E $_2$), progesterone, or vitamin D $_3$, respectively. The plates were incubated at 30 °C for 5 days. Specific interactions were measured by the appearance of yeast colonies on SD-Ade-Leu-Trp plates and a liquid β -gal assay. The data represent the mean \pm S.D. of three independent colonies. *B*, CV-1 cells were transiently transfected with 100 ng of pMMTV-Luc, 50 ng of pSV40- β -gal, 10 ng of pSV-AR, pSV-GR, or pSV-PR β as indicated, and 60 ng of pcDNA3 vector or pcDNA3-FLAG- β -catenin. Cells were incubated with or without 10 nM DHT, dexamethasone, or progesterone, respectively. Cell lysates were measured for luciferase and β -gal activities.

β -Catenin Augments AR-mediated Transcription through Specific Protein-Protein Interaction—Transient transfection assays were performed to further investigate the possible effect of β -catenin on AR-mediated transcription. Plasmids capable of expressing AR, wild type or mutants of β -catenin, and a luciferase reporter plasmid regulated by the MMTV-LTR (MMTVpA3-Luc), were transfected into CV-1 cells (35). A nearly 3-fold ligand-dependent transactivation was observed in the cells transfected with AR plasmid alone. Cotransfection of the wild type of β -catenin expression construct increased AR activity to nearly 10-fold above base line (Fig. 6A). Expression of the β -catenin mutant lacking the armadillo repeat 12 still showed 6–7-fold induction, whereas the mutants lacking repeat 6 showed no enhancement on AR-mediated transcription (Fig. 6A). These data indicate that β -catenin augments AR-mediated transcription, and this enhancement is mediated through the physical interaction between these proteins.

β -Catenin can form a transcriptional complex with members of the Tcf/LEF family to activate target genes (7, 25). To ensure that augmentation of the MMTV promoter by β -catenin is mediated solely through the AR, rather than through other transcription factors, we examined the effect of β -catenin on

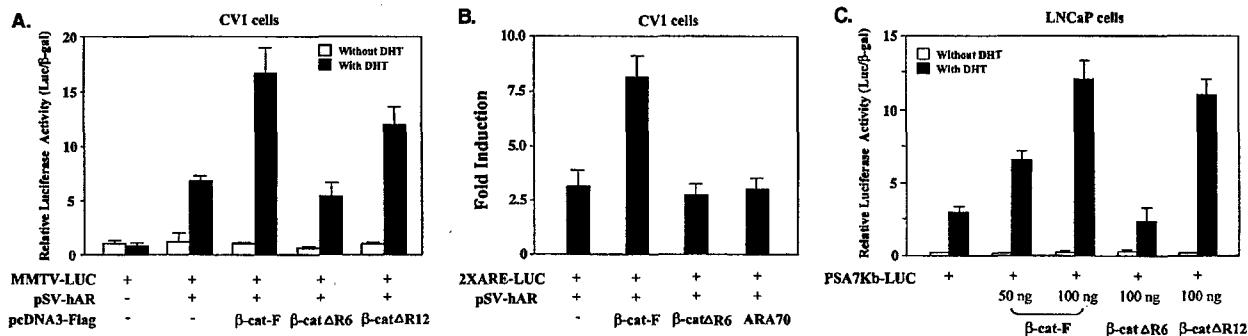


FIG. 6. β -Catenin enhances AR-mediated transcription. *A*, CV-1 cells were transfected with MMTV-luc reporter (100 ng), pcDNA3- β -gal (25 ng), pSV-hAR (5 ng), and the wild type and mutants of pcDNA3-FLAG- β -catenin (100 ng) as indicated. Twenty-four hours after transfection, cells were incubated with or without 10 nM DHT for 24 h. Cell lysates were prepared for assessment of luciferase and β -gal activities (as controls of transfection efficiency). *B*, similar to *A*, except that a 2XARE-luc reporter (100 ng) was used. *C*, LNCaP cells were transfected with PSA7kb-luc reporter (100 ng), pcDNA3- β -gal (25 ng), and the wild type and mutants of pcDNA3-FLAG- β -catenin as indicated. Twenty-four hours after transfection, cells were treated with or without 10 nM DHT for 24 h. Cell lysates were measured for luciferase and β -gal activities. The data represent the mean \pm S.D. of three independent samples.

the transcription from a luciferase reporter driven by a minimum promoter with two androgen response elements (AREs). A similar ligand-dependent enhancement of AR-mediated transcription was observed with the full-length β -catenin construct (Fig. 6*B*). As we observed above, the mutant lacking repeat 6 showed no enhancement. Interestingly, ARA70, an AR coactivator, did not affect the AR-mediated transcription of this minimum promoter. Nevertheless, these results further confirm that β -catenin is truly a coactivator of AR and can enhance AR-mediated transcription on a minimum promoter with AREs.

To evaluate the enhancement by β -catenin of AR-mediated transcription in a physiologically relevant cellular context, an AR-positive prostate cancer cell line, LNCaP, was transfected with β -catenin expression constructs and a luciferase reporter driven by the 7-kb prostate-specific antigen (PSA) gene promoter, which is an AR-regulated target gene and has been widely used as a prostate-specific tumor marker (36). As seen in Fig. 6*C*, the wild type and repeat 12 deletion mutants of β -catenin enhance endogenous AR-mediated transcription from the PSA promoter, and the wild type β -catenin showed a dose-dependent effect. However, as we observed previously, the mutant with a deletion of repeat 6 showed no effect. These data further support the transfection data with MMTV and ARE-minimum promoters and demonstrate that the augmentation of endogenous AR activity by β -catenin in prostate cancer cells is mediated through the AR/ β -catenin interaction.

E-cadherin Modulates the Level of Cytoplasmic Pools of β -Catenin to Enhance AR-mediated Transcription—The observation that β -catenin can function as an oncogene when inappropriately expressed highlights the importance of regulating β -catenin level in the cells. Recent studies show that tumor cells can bypass this regulation by acquiring loss-of-function mutations in components of the destruction complex or by altering regulatory sequences in β -catenin itself, which makes it impervious to the effects of the destruction complex. Moreover, in normal epithelial tissues, E-cadherin complexes with the actin cytoskeleton via catenins to maintain the functional characteristics of epithelia (37, 38). Disruption of this complex, due primarily to loss or decreased expression E-cadherin, is frequently observed in many advanced, poorly differentiated prostate cancer patient samples (39, 40). It has been reported that β -catenin is mainly accumulated in both the cytoplasm and the nucleus of some prostate cancer cell lines in which there is a reduction or loss of E-cadherin expression (41, 42).

To test whether loss of E-cadherin can augment AR activity by increasing cytoplasmic and nuclear levels of β -catenin, we

stable transfected E-cadherin expression vectors into an E-cadherin-negative prostate cancer cell line, TSU.pr-1. In TSU.pr-1 cells, E-cadherin expression is silenced by hypermethylation of the promoter region (41). Immunostaining of the polyclonal subline transfected with the E-cadherin expression vector (TSU.pr-1/E-CAD) showed that β -catenin is partially redistributed into the cell membrane, resulting in reduction of its cytoplasmic and nuclear levels compared to the pool transfected with a control vector, TSU.pr-1/Neo (Fig. 7*A*). Transfection of an AR expression vector and the PSA-luciferase reporter into these two TSU.pr-1 sublines showed a ligand-dependent AR activity (Fig. 7*B*). However, a stronger AR activity was observed in TSU.pr-1/Neo cells than TSU.pr-1/E-CAD cells with equal amounts of AR plasmid, and a dose-dependent induction was only shown in TSU.pr-1/Neo cells. Using an ARE-luciferase reporter, we also showed a similar dose-dependent augmentation of AR activity by the cytoplasmic pool of endogenous β -catenin in TSU.pr-1/Neo cells (Fig. 7*E*). The results from the above experiments suggest that endogenous β -catenin in the cytoplasmic pool can augment AR-mediated transcription and that reducing its level can decrease this enhancement.

To further confirm that the enhancement of AR activity in TSU.pr-1/Neo cells was directly mediated by β -catenin, we repeated the above experiments with an antisense construct of β -catenin. As shown in Fig. 7*C*, enhancement of ligand-dependent AR activation in TSU.pr-1/Neo cells was specifically repressed by cotransfection with the β -catenin antisense construct. This was correlated with a decreased level of β -catenin protein in the cells (Fig. 7*D*). To ensure that the enhancement of AR activity in TSU.pr-1/Neo cells was specifically mediated by endogenous β -catenin, rather than a general effect on the basal transcriptional machinery or other nonspecific effects from this subline, we examined the transcriptional activities of other nuclear hormone receptors in the cells. As shown in Fig. 7*F*, unlike the results that we observed in Fig. 7*E*, ER α , PR β , and VDR showed no significant differences in ligand-dependent activities between TSU.pr-1/Neo and TSU.pr-1/E-CAD cells. These results are consistent with our yeast data showing that β -catenin selectively interacts with AR. Taken together, we conclude that overexpression of E-cadherin in TSU.pr-1 induces a redistribution of the cellular localization of β -catenin protein, which can directly affect AR-mediated transcription.

DISCUSSION

The nuclear receptor superfamily coordinates the complex events involved in development, differentiation, and physiological response to diverse stimuli. Transcriptional activity of the

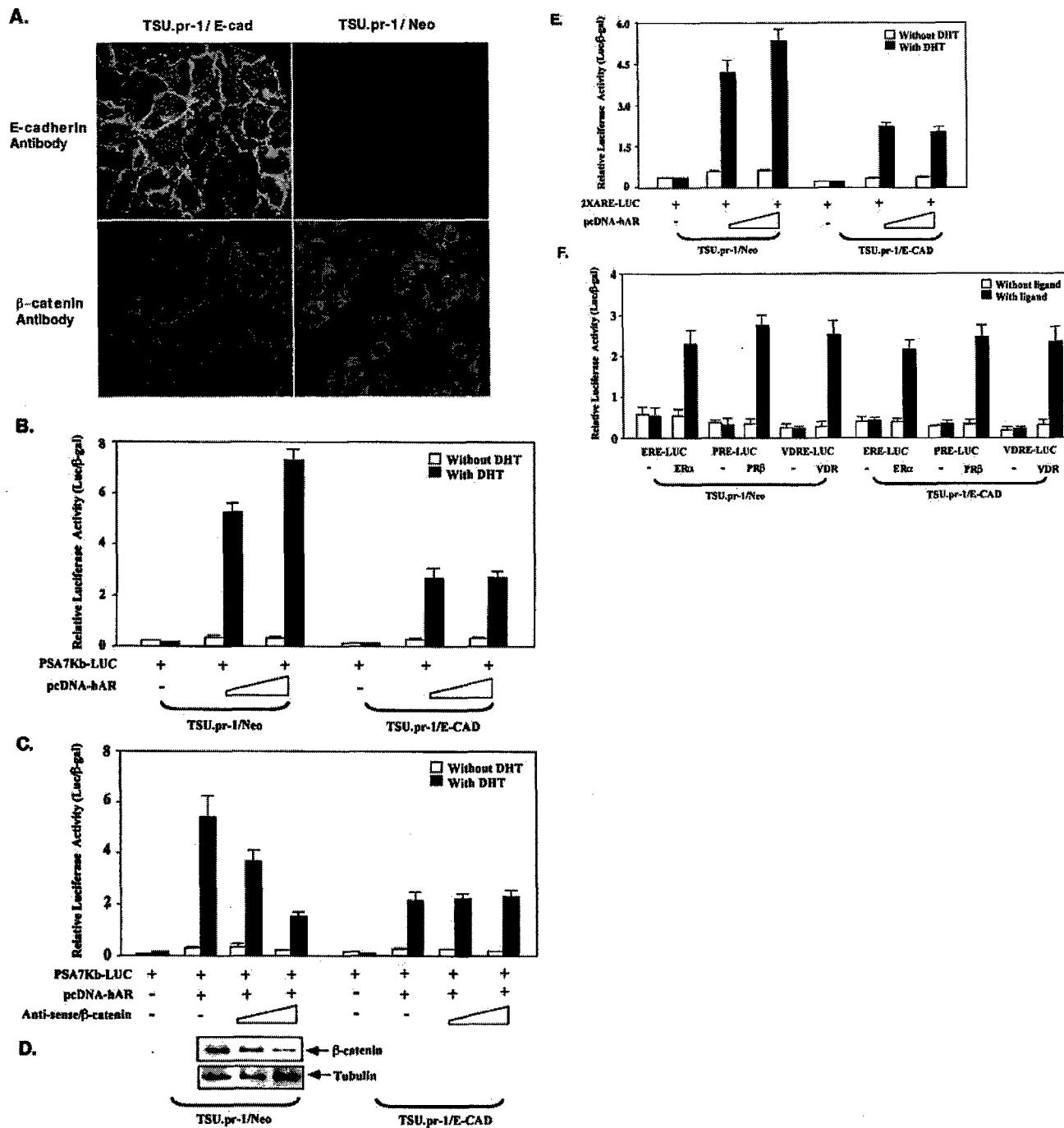


FIG. 7. *β*-Catenin augments AR-mediated transcription in human prostate cancer cells. *A*, two TSU.pr-1 sublines stably transfected with an E-cadherin expression plasmid (TSU.pr-1/E-CAD) or a control vector, pcDNA3 (TSU.pr-1/Neo), were stained with E-cadherin (green) and *β*-catenin (red) antibodies. *B*, 100 ng of PSA7Kb-Luc alone or with 10 or 30 ng of pcDNA-hAR were transfected into the TSU.pr-1 cells. White bars represent the absence of DHT; black bars represent the addition of 10 nM DHT. Luciferase activity is reported as relative light units and is represented as mean \pm S.D. *C*, an antisense construct of *β*-catenin (20 and 60 ng) was cotransfected with the PSA-luc reporter and AR expression plasmid. Relative luciferase activities were measured. *D*, total cell lysates isolated from the above experiment were analyzed by Western blotting to determine the cellular levels of *β*-catenin proteins. *E*, a luciferase reporter driven by two AREs (100 ng) and an AR expression vector (10 or 30 ng) were transfected into TSU.pr-1/NEO and TSU.pr-1/E-CAD cells. *F*, for each sample, 25 ng of pSV-*β*-gal, 100 ng of luciferase reporter vectors containing the different hormone response elements such as pERE-luc, pPRE-luc, and pVDRE-luc, and 10 ng of the corresponding receptor expression constructs were transfected. The specific ligands for each receptor were added for induction, and these included 10 nM β -estradiol, progesterone, and 1 α ,25-dihydroxyvitamin.

nuclear hormone receptors can be modulated by coactivators and corepressors (43). Aberrations of these cofactors may lead to enhancement of receptor activity to provide an adaptive advantage for cell growth. Changes in the transcriptional programs of nuclear receptors such as the AR are important but poorly understood events in tumor development and progres-

sion. The experiments reported here demonstrate a specific protein-protein interaction between AR and *β*-catenin. Further characterization of the interaction in the yeast two-hybrid assays and in *in vitro* GST-pull down experiments showed that the LBD of AR is necessary and sufficient for the interaction with *β*-catenin in an androgen-dependent manner. This sug-

gests that conformational changes in the LBD of AR, following binding to ligand, are necessary for the interaction of β -catenin with AR protein. Using immunofluorescence studies, we first demonstrated that β -catenin can translocate into the nucleus as part of a complex with AR. These multiple lines of evidence clearly indicated that AR and β -catenin proteins can specifically interact and given the consequences of the interaction, probably represents a biologically relevant interaction.

In this study, we also demonstrated a functional interaction between AR and β -catenin in prostate cancer cells, which is consistent with a recent report by Truica *et al.* (31). Importantly, using internal deletion mutants of β -catenin, we showed that enhancement of intact, natural AR-dependent promoters from MMTV and the PSA gene by β -catenin can be completely abolished by deleting armadillo repeat 6 of the protein. Similar results were obtained with a mini-promoter containing only two androgen response elements. These results provide the first line of evidence demonstrating that augmentation of AR activity by β -catenin is mediated solely through a specific protein-protein interaction. These results further support the possibility that the AR/ β -catenin interaction characterized in this study is biologically relevant.

As a key player in both cell-cell adhesion and Wnt signaling, β -catenin is regulated by multiple signaling pathways through binding to other protein partners, including Tcf/LEF family members, axin, APC, and catenins (44, 45). The crystal structure of the armadillo domain of β -catenin revealed that each of the armadillo repeats consists of three α -helices, and together the 12 repeats form a superhelix (29). This unique structure provides a long positively charged groove for binding. It has been shown that most of the β -catenin-binding proteins bind β -catenin mainly through the central armadillo domain (29, 30). The binding regions overlap, but in general, a minimum of 6–7 repeats is sufficient for detectable binding. Using a series of deletion mutants, we demonstrated that the NH₂ terminus and first six armadillo repeats of β -catenin are primarily responsible for binding to AR. Significantly, multiple lines of evidence from this study showed that a deletion lacking repeat 6 can completely abolish the binding, which suggests this region may directly form an interface with AR. Although most of β -catenin-interacting proteins bind β -catenin in the central armadillo domain, each of these proteins may bind to β -catenin differently depending on divergent binding regions. Further structural studies of AR/ β -catenin interaction should lead to important information that may provide the basis for designing compounds to block this interaction.

A number of cofactors have been identified that interact with the LBD of nuclear hormone receptors (22, 46–48). Among them, the best characterized coactivators are the p160 family and p300/CBP, which appear to bind to most of the nuclear receptors in a ligand-dependent manner through the conserved protein motif, LXXLL (49, 50). The motif forms a two-turn amphipathic α -helix, which binds to a hydrophobic cleft in the activation domain 2 of nuclear receptors. β -Catenin contains five LXXLL motifs, all of which are located in a central armadillo domain (29). Among them, four are localized in the second helix of the armadillo repeats 1, 7, 10, and 12. Based on the structure of β -catenin protein, the Leu residues in these motifs are buried in the hydrophobic core of the armadillo repeats, and it seems unlikely that they would interact with AR or other nuclear hormone receptors (29). Using a series of deletion mutants, we have shown that constructs lacking the repeats 7, 10, and 12 retained the capacity to bind AR, whereas the construct lacking only repeat 6 fully abolished the interaction. Under identical experimental conditions, other steroid hormone receptors such as ER α , PR β , and GR did not show an interaction

with β -catenin. These data are consistent with an earlier structural study of β -catenin and suggest that the LXXLL motifs in β -catenin may not directly contribute to the interaction that we have identified between AR and β -catenin. An earlier report showed that β -catenin associates with a retinoic acid receptor and enhance activation of a retinoic acid-responsive promoter (51). In the yeast two-hybrid system, we also observed a weak interaction between β -catenin and VDR. In this regard, it will be important to determine the protein motifs involved in the interaction with these receptors, which will expand our understanding of the cross-talk between β -catenin and nuclear hormone receptors.

The cellular levels of β -catenin are tightly regulated in normal cells. Mutations affecting the degradation of β -catenin can increase the cellular levels of the proteins to induce neoplastic transformation (52). A tumor suppressor, APC, which is an important component of the degradation machinery, was frequently mutated in both sporadic and hereditary colorectal tumors (12). Mutations of β -catenin within the GSK binding region were also found in prostate cancer samples (53), suggesting a potential role of β -catenin in prostate cancer cells. Our results showing a detailed molecular basis of the interaction of β -catenin with AR provide a direct link between β -catenin and androgen signaling. Due to an abnormal cadherin-catenin interaction in the cell membrane, increasing the cytoplasmic and nuclear levels of β -catenin as a consequence of loss of E-cadherin is frequently observed in late stages of prostate cancer cells (15). Using an E-cadherin-negative prostate cancer cell line, TSU.pr-1, we further showed that the endogenous β -catenin that accumulated in cytoplasm and nucleus are capable of augmenting AR-mediated transcription, and the effect of β -catenin on AR can be enhanced by loss of E-cadherin expression. These results suggest that loss of E-cadherin expression may promote AR-mediated cell growth in late stages of prostate cancer. In addition, as observed previously (16), β -catenin was shown to have no effect with a TCF reporter gene in TSU.pr-1 cells (data not shown). A similar observation was also reported recently in breast cancer cells containing transcriptional silencing of the E-cadherin gene (54). This raises the question as to whether the growth-promoting effect of β -catenin is mediated through other partners rather than through the TCF/LEF pathway in prostate cancer or/and other tumor cells.

Our results suggest a new role for E-cadherin and β -catenin in prostate cancer cells. During prostate cancer progression, loss of expression of E-cadherin frequently occurs, which leads to an increase in the cytoplasmic levels of β -catenin. Under normal conditions, the cellular β -catenin is tightly regulated by the destruction complex which includes APC, GSK3 β , and axin. When the functional activities of these components are changed, such as by mutation or aberrant expression of the proteins, the excessive free β -catenins overload the system and are translocated into the nucleus, where they specifically interact with the AR to augment AR-mediated transcription. In addition, enhancement by β -catenin may also be able to maintain or increase AR activity in the setting of decreased androgen levels during androgen ablation therapy, which can adapt prostate cancer cells to become androgen insensitive. Therefore, studying the interaction of β -catenin with AR in prostate cancer should provide fresh insight into the progression of prostate cancer that may help us to identify new steps that can be targeted for prostate cancer treatment.

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Phosphatidylinositol 3-Kinase/Akt Stimulates Androgen Pathway through GSK3 β Inhibition and Nuclear β -Catenin Accumulation*

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PI3K/Akt plays a critical role in prostate cancer cell growth and survival. Recent studies have shown that the effect of PI3K/Akt in prostate cells is mediated through androgen signaling. The PI3K inhibitor, LY294002, and a tumor suppressor, PTEN, negatively regulate the PI3K/Akt pathway and repress AR activity. However, the molecular mechanisms whereby PI3K/Akt and PTEN regulate the androgen pathway are currently unclear. Here, we demonstrate that blocking the PI3K/Akt pathway reduces the expression of an endogenous AR target gene. Moreover, we show that the repression of AR activity by LY294002 is mediated through phosphorylation and inactivation of GSK3 β , a downstream substrate of PI3K/Akt, which results in the nuclear accumulation of β -catenin. Given the recent evidence that β -catenin acts as a coactivator of AR, our findings suggest a novel mechanism by which PI3K/Akt modulates androgen signaling. In a PTEN-null prostate cancer cell line, we show that PTEN expression reduces β -catenin-mediated augmentation of AR transactivation. Using the mutants of β -catenin, we further demonstrate that the repressive effect of PTEN is mediated by a GSK3 β -regulated degradation of β -catenin. Our results delineate a novel link among the PI3K, wnt, and androgen pathways and provide fresh insights into the mechanisms of prostate tumor development and progression.

Prostate cancer is the most common malignancy in men and the second leading cause of cancer death in the United States (1). The fact that androgen ablation is an effective treatment for the majority of prostate cancers indicates that androgen plays an essential role in regulating the growth of prostate cancer cells (2, 3). The growth-promoting effects of androgen in prostate cells are mediated mostly through the androgen receptor (AR).¹ The AR belongs to the nuclear receptor superfamily and acts as a ligand-dependent transcription factor (4, 5). Recent studies suggest that other signal transduction pathways can modulate AR activity and that they may also contribute to the development and progression of prostate cancer (6, 7).

The phosphatidylinositol 3-kinase (PI3K) consists of regula-

tory (p85) and catalytic (p110) subunits that participate in multiple cellular processes including cell growth, transformation, differentiation, and survival (8). An oncoprotein, Akt/PKB, has been identified as a key effector of the PI3K signaling pathway (9, 10). The binding of PI3K-generated phospholipids to Akt results in the translocation of Akt from the cytoplasm to the inner surface of the plasma membrane where Akt is phosphorylated by the upstream kinases, PDK-1, PDK-2, and ILK (11, 12). The activation of Akt results in the phosphorylation of a number of downstream substrates such as glycogen synthase kinase (GSK3), Bad, and caspase9 and the forkhead transcription factors, Raf, I κ b kinase, and phosphodiesterase 3B (13). As one of the principal physiological substrates of Akt, GSK3 is a ubiquitously expressed protein serine/threonine kinase that was initially identified as an enzyme that regulates glycogen synthesis in response to insulin (14, 15). It has been shown that GSK3 β plays an important role in the Wnt pathway by regulating the degradation of β -catenin (16, 17).

β -catenin plays a pivotal role in cadherin-based cell adhesion and in the Wnt signaling pathway (18). Corresponding to its dual functions in cells, β -catenin is localized to two cellular pools. Most of the β -catenin is located in the cell membrane where it is associated with the cytoplasmic region of E-cadherin, a transmembrane protein involved in homotypic cell-cell contacts (19). A smaller pool of β -catenin is located in both the nucleus and cytoplasm where it mediates Wnt signaling. In the absence of a Wnt signal, β -catenin is constitutively down-regulated by a multicomponent destruction complex containing GSK3 β , axin, and the tumor suppressor adenomatous polyposis coli. These proteins promote the phosphorylation of serine and threonine residues in the amino-terminal region of β -catenin and thereby target it for degradation by the ubiquitin proteasome pathway (20). Wnt signaling inhibits this process, which leads to an accumulation of β -catenin in the nucleus and promotes the formation of transcriptionally active complexes with members of the Tcf/LEF family (21) and other transcription factors (22, 23).

The tumor suppressor PTEN is a phosphoprotein/phospholipid dual specificity phosphatase (24). Early studies indicated that somatic mutation of *PTEN* is a common event in a variety of human tumors including prostate cancer (25). *PTEN* was found to be mutated in primary prostate tumors, metastatic prostate cancers, and in prostate cancer cell lines (25, 26). In addition, the reduced expression of PTEN protein as well as increased Akt activity has been observed in xenograft models (27). Recently, it has been shown that PTEN inhibits PI3K/Akt-stimulated androgen-promoted cell growth and AR-mediated transcription in prostate cancer cells (28).

PI3K/Akt has been shown to promote prostate cancer cell survival and growth via enhancing AR-mediated transcription. Both PTEN and the PI3K inhibitor LY294002 negatively regulate this process (28, 29). Although several potential mecha-

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¹ The abbreviations used are: AR, androgen receptor; PI3K, phosphatidylinositol 3,4,5-trisphosphate; GSK3 β , glycogen synthase kinase 3 β ; PTEN, phosphatase and tensin homolog deleted on chromosome 10; DHT, dihydrotestosterone; PSA, prostate-specific antigen.

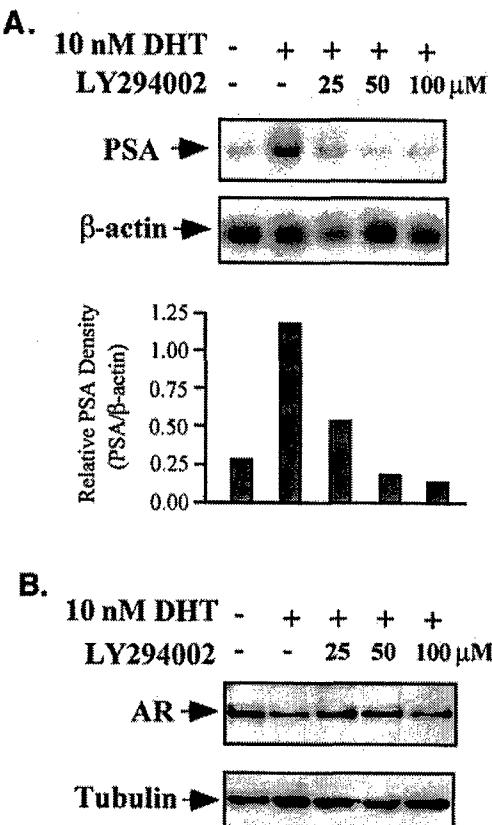


FIG. 1. The PI3K inhibitor represses AR-mediated transcription. *A*, total RNAs were isolated from LNCaP cells cultured in T-medium with or without 10 nM DHT, treated for 4 h with the PI3K inhibitor LY294002 or vehicle, and analyzed by Northern blotting. Expression of the endogenous PSA gene was detected by a cDNA probe derived from the human PSA gene. A β-actin probe was used to confirm equal RNA loading. Densitometry of the membrane blot was performed, and the relative numbers were reported as optical density units of PSA/β-actin (PSA/β-actin). *B*, whole cell lysates were isolated from LNCaP cells treated as described above and analyzed by Western blotting to detect the expression of AR and tubulin proteins.

nisms have been suggested for this cross-talk, the precise molecular basis by which PI3K/Akt and PTEN regulate AR-mediated transcription is currently unclear. Recently, a specific protein-protein interaction between β-catenin and AR was identified by us and others (22, 23). Through this interaction, β-catenin augments the ligand-dependent activity of AR in prostate cancer cells. Here, we provide multiple lines of evidence showing that the cross-talk between the androgen and PI3K/Akt pathways is mediated through the modulation of the PI3K/Akt downstream effector GSK3β. Its inactivation by phosphorylation results in increased nuclear levels of β-catenin, which augment AR activity. These findings delineate a novel mechanism by which PI3K/Akt and PTEN regulate the androgen pathway during prostate cell growth and survival.

EXPERIMENTAL PROCEDURES

Cell Cultures and Transfections—An AR-positive prostate cancer cell line LNCaP was maintained in T-medium (Invitrogen) with 5% fetal calf serum. Transient transfections were carried out in RPMI 1640 medium using LipofectAMINE 2000 (Invitrogen) as described previously (23). In the experiments with the PI3K inhibitor LY294002 (Alexis, San Diego, CA), cells were usually cultured for 16 h and then were treated with different concentrations of the inhibitor in Me_2SO or vehicle only for 20 min to 2 h. For androgen induction experiments, cells were grown in T-medium with charcoal-stripped fetal calf serum (Hyclone, Denver, CO) for 14 h and treated with 10 nM DHT in ethanol and different concentrations of LY294002 for 4 h.

Northern Blot Analysis—Total RNAs were isolated from LNCaP

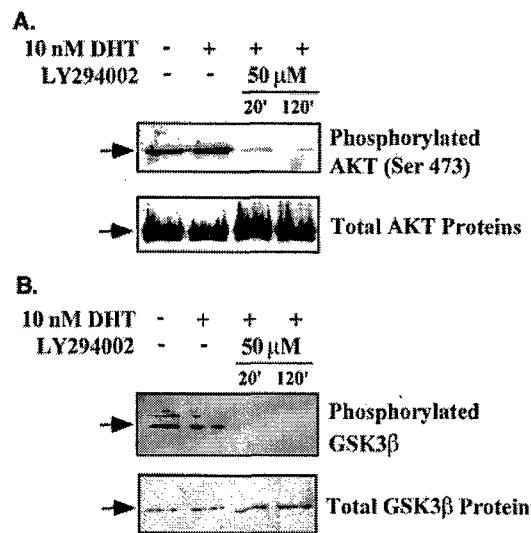


FIG. 2. Inhibition of Akt and GSK3β phosphorylation by LY294002 in prostate cancer cells. Whole cell lysates were isolated from LNCaP cells that were treated as indicated in Fig. 1 and “Experimental Procedures,” and were analyzed by Western blotting. Both total and phosphorylated Akt (A) and GSK3β (B) were detected by specific antibodies as indicated in the figure.

cells treated with LY294002 for 4 h in the presence of 10 nM DHT in ethanol or vehicle alone using an RNAzit kit (Ambion, Austin, TX). For Northern blotting, 5 μ g of total RNA were electrophoresed on a 1% agarose-formaldehyde gel, transferred to Hybond-N nylon membranes (Amersham Biosciences) by capillary blotting in 20 \times SSC, and hybridized with a DNA fragment (amino acids 1–261) derived from the human prostate-specific antigen (PSA) gene. The blots were stripped and rehybridized with a β-actin probe (30).

Preparation of Whole Cell and Nuclear Extracts—LNCaP cells were cultured in duplicate flasks to collect both whole cell lysates and nuclear extracts. To make the whole cell lysates, cells were washed with phosphate-buffered saline and were resuspended in RIPA buffer (1% Nonidet P-40, 0.1% SDS, 50 mM NaF, 0.2 mM Na_3VO_4 , 0.5 mM dithiothreitol, 150 mM NaCl, 2 mM EDTA, 10 mM sodium phosphate buffer, pH 7.2). Nuclear extracts were prepared from LNCaP cells essentially according to the method of Dignam *et al.* (31) with minor modifications. The cells were washed with phosphate-buffered saline and mechanically disrupted by scraping into homogenization buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl_2 , 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride) and incubated on ice for 10 min. Cells were further disrupted by 10 strokes of a homogenizer and centrifuged at 15,000 rpm for 20 min. The pellet was resuspended in buffer containing 20 mM Hepes, pH 7.9, 420 mM NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 25% glycerol and then homogenized with 10 strokes. The lysate was incubated on ice for 30 min and centrifuged for 10 min at 15,000 rpm. The supernatant was saved and analyzed as the nuclear fraction.

To prepare the cytosolic fraction, LNCaP cells treated with LY294002 were lysed in digitonin lysis buffer (1% digitonin, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2). The lysates were centrifuged at 13,000 rpm for 10 min, and the supernatants were saved as cytosolic components. The pellets representing cytoskeletal and nuclear components were lysed in RIPA buffer.

SDS-PAGE and Immunoblotting—Protein fractions for immunoblotting were boiled in SDS sample buffer and then resolved on a 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and probed with appropriate antibodies including an anti-human Akt (provided by Dr. Richard Roth, Stanford University, Stanford, CA), phospho-Akt-(Ser-473) (catalog number 9271, Cell Signaling Technology, Beverly, MA), phospho-GSK3α/β-(Ser-21/Ser-9) (catalog number 9331, Cell Signaling Technology), AR (catalog number sc-816, Santa Cruz Biotechnology, Santa Cruz, CA), Sin3A (catalog number sc-996, Santa Cruz Biotechnology), tubulin (catalog number MS-581-P, Neomarker, Fremont, CA), β-catenin (catalog number C19220, Transduction Laboratories, Lexington, KY), and GSK3β (catalog number G22320, Transduction Laboratories). Proteins were detected using the ECL kit (Amersham Biosciences). The nuclear fractions were analyzed by SDS-PAGE. Equal loading of the nuclear proteins was ascertained

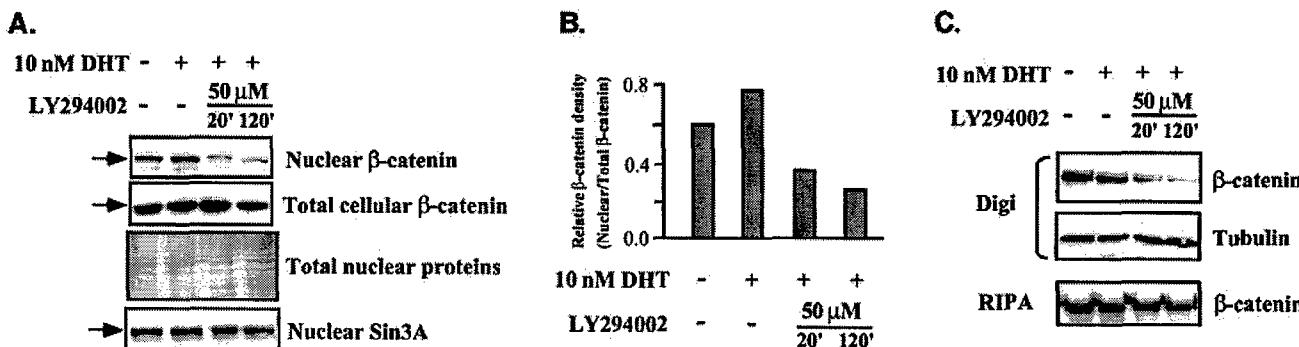


Fig. 3. Inhibition of PI3K signaling results in decreased nuclear accumulation of β -catenin in prostate cancer cells. *A*, both nuclear extracts and whole cell lysates were isolated from LNCaP cells treated with LY294002 and DHT and resolved by SDS-PAGE. The β -catenin and Sin3A antibodies were used for the detection of protein expression. The same membrane used for the Western blotting was also stained with Ponceau S stain solution for measuring equal protein loading. *B*, densitometry of nuclear β -catenin proteins is shown as relative β -catenin density (optical density units of nuclear proteins/optical density units of total proteins). *C*, both cytosolic fraction (*Digi*) and cytoskeletal fraction (*RIPA*) were prepared from LNCaP cells as described under "Experimental Procedures" and were analyzed by Western blotting. Both β -catenin and tubulin were detected using specific antibodies.

by reversible staining with the Ponceau S solution (Catalog number P7767, Sigma).

Plasmid Construction—The pcDNA3-AR expression vector was generated in the laboratory and used for the transient transfection experiments. Expression constructs of human PTEN were generously provided by Dr. William Sellers (Dana-Farber Cancer Institute, Boston, MA) and used for subcloning into the pCMV5 vector. PLNCX-HA-myR-AKT and PLNCX-HA-myR-AKT179M were also kindly provided by Dr. Sellers (32). The reporter plasmid pPSA7kb-luc with the luciferase gene under the control of promoter fragments of the human prostate-specific antigen was provided by Dr. Jan Trapman (33). The mutants of β -catenin with a single point mutation in the GSK3 β phosphorylation sites were generated by a PCR-based mutagenesis scheme. The key serine amino acid residues were mutagenized by using sets of primers containing two or three nucleotide changes in conjunction with upstream and downstream primers. The appropriate fragments with in-frame restriction enzyme sites were generated by PCR, cleaved with restriction enzymes, and cloned into the pcDNA3 vector (Invitrogen). All of the constructs were sequenced from both ends of the inserts to confirm that no extraneous mutations were introduced by PCR.

Luciferase and β -Galactosidase Assay—Luciferase activity was measured in relative light units as described previously (30). 50 μ l of cell lysate was used for luciferase assays. The light output is measured after a 5-s delay following injection of 50 μ l of luciferase buffer and 50 μ l of Luciferin by the dual injector luminometer according to manufacturer's instruction (Analytical Luminescence Laboratories, San Diego, CA). The relative light units from individual transfections were normalized by the measurement of β -galactosidase activity expressed from a cotransfected plasmid in the same samples. Individual transfection experiments were done in triplicate, and the results are reported as the luciferase/ β -galactosidase mean \pm S.D. from representative experiments.

RESULTS

Inhibition of the PI3K/AKT Pathway Represses AR-mediated Transcription—PI3K/Akt enhances the activity of AR-regulated reporter genes in transient transfection experiments (28, 29). To evaluate the effect of PI3K/Akt on AR-mediated transcription in a physiologically relevant cellular context, we examined the expression of the endogenous PSA gene in an AR-positive prostate cancer cell line LNCaP treated with the PI3K inhibitor LY294002. In the presence of 10 nM DHT, PSA expression was increased \sim 4-fold in LNCaP cells over that found in cells not treated with DHT (Fig. 1A). At concentrations of LY294002 from 25–100 μ M, the expression of PSA was significantly reduced. An \sim 4-fold reduction of PSA transcripts was found in the cells treated with 100 μ M LY294002 using the level of β -actin transcripts as an internal control (Fig. 1A). Low concentrations (5 μ M) of LY294002 induced only a slight reduction of PSA expression during a 4-h treatment but showed a significant reduction of PSA expression after 16 h (data not shown). To ensure that this repression was not the result of

LY294002-induced changes in the intracellular steady-state levels of AR protein, we examined both the AR and tubulin protein levels in the cell samples used for the Northern blotting. We found that there was no significant change in protein expression (Fig. 1B). This result provided the first line of evidence that inhibition of PI3K/Akt could suppress endogenous AR-mediated transcription in prostate cancer cells.

Repression of the PI3K/AKT Pathway Inhibits Phosphorylation of GSK3 β and Nuclear Accumulation of β -catenin in Prostate Cancer Cells—To further elucidate the mechanism by which LY294002 inhibits endogenous AR transactivation in LNCaP cells, we first assessed the phosphorylation state of Akt. It has been reported that PDK-1 phosphorylation of threonine 308 in the activation loop of the catalytic domain of Akt allows autophosphorylation of serine 473 (a hydrophobic phosphorylation site) in the carboxyl terminus (34). To demonstrate that the effect of LY294002 on PSA transcription was attributed to inhibition of Akt, we evaluated Akt activation using a phosphorylation-specific antibody for Ser-473. As shown in Fig. 2A, the phosphorylation of Akt proteins was significantly inhibited by LY294002 in LNCaP cells, even after a very short pulse (20 min). In contrast, the total amount of Akt protein showed no differences in the presence or absence of LY294002.

Because GSK3 β is one of the major downstream targets of Akt, we next assessed whether LY294002 also affected the phosphorylation of GSK3 β . Using specific antibodies, we examined both the total and phosphorylated GSK3 β proteins in the same cell samples used for detecting Akt. As expected, the phosphorylation of GSK3 β proteins was also significantly impaired by treatment with LY294002, whereas almost equal amounts of total GSK3 β proteins were found in both treated and untreated cells (Fig. 2B). At either 5 or 20 μ M LY294002, we observed a similar inhibitory effect on the phosphorylation of both Akt and GSK3 β in cells treated for 12 h (data not shown). Taken together, the results demonstrate that the suppression of the PI3K pathway by the PI3K inhibitor LY294002 blocks the phosphorylation of both Akt and GSK3 β proteins in LNCaP cells.

The above data demonstrate that the treatment of LNCaP cells with LY294002 results in a decreased level of expression of the endogenous PSA gene and an inhibition of the phosphorylation of Akt and GSK3 β . It has been shown that GSK3 β regulates the cellular levels of β -catenin by targeting it to the ubiquitin proteasome pathway via the destruction complex (20). Previous studies have shown that inactivation of GSK3 β by phosphorylation can induce the nuclear accumulation of β -catenin because of decreased degradation (17, 35). To eval-

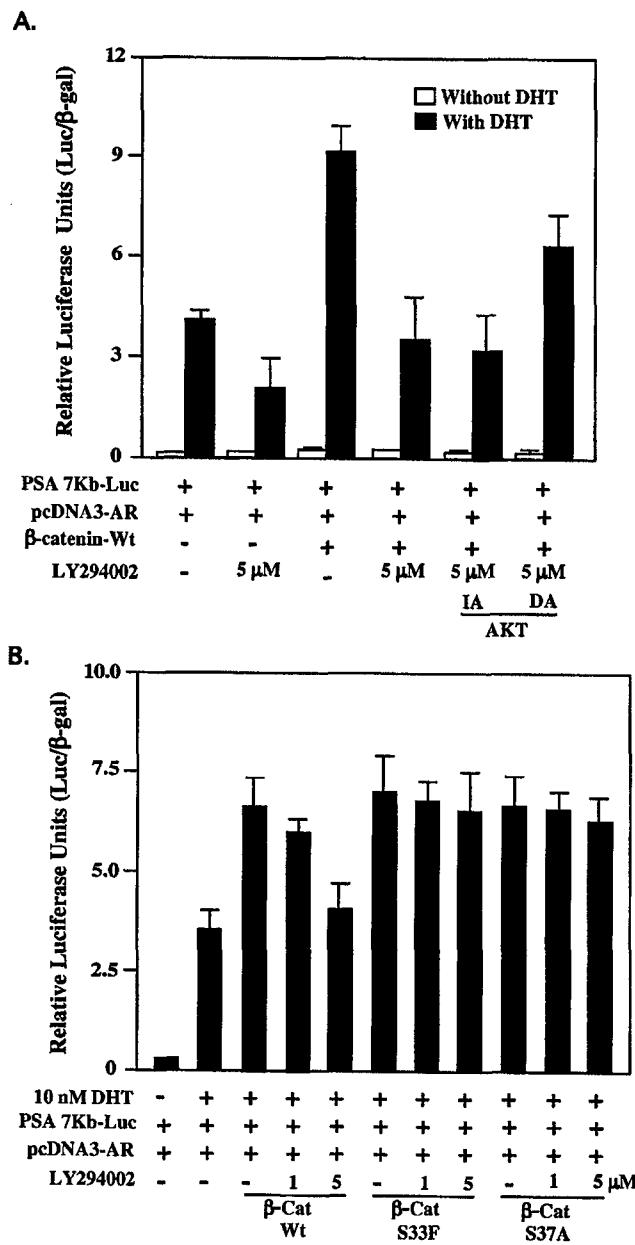


FIG. 4. Inhibition of AR activity by LY294002 is mediated through Akt and GSK3 β . *A*, transient transfections were performed in LNCaP cells with 100 ng of PSA7kb-luc reporter, 5 ng of pcDNA3-AR, 25 ng of pcDNA3- β -galactosidase, and 50 ng of wild type pcDNA3-FLAG- β -catenin in the presence or absence of 10 nM DHT for 18 h. Cell lysates were measured for luciferase and β -galactosidase activities. The data represent the mean \pm S.D. of three independent samples. *B*, LNCaP cells were cotransfected with 50 ng of wild type β -catenin or the mutants of β -catenin containing a point mutation within the GSK3 β binding site as well as with the other plasmids indicated in the figure. The cells were treated with DHT and LY294002 as described above.

ate the downstream effect of GSK3 β in LNCaP cells, we next examined the nuclear levels of β -catenin. Nuclear extracts and whole cell lysates were prepared from cells that were treated with LY294002 or with vehicle only. As shown in Fig. 3A, there was no significant change in the amount of total β -catenin protein in the treated compared with the untreated cells. However, there was a 2–3-fold reduction in nuclear β -catenin in the

cells treated with LY294002 (Fig. 3, *A* and *B*). In contrast, the controls, total nuclear protein, and the transcriptional repressor Sin3A showed no change (Fig. 3A). To confirm these findings, we examined the level of free cytosolic β -catenin protein in LNCaP cells treated with LY294002 (36). As shown in Fig. 3C, after LY294002 treatment, free β -catenin in the cytosolic compartment (Digi) was significantly reduced, whereas β -catenin in the cytoskeletal compartment (RIPA) remained unchanged. Taken together, these results demonstrate that blocking PI3K signaling results in a decrease in both the free cytosolic and nuclear β -catenin in prostate cells.

Repression of AR Activity by LY294002 Is Mediated through the Downstream Effectors of PI3K, Akt, and GSK3 β —To further study the repressive effect of LY294002 on AR-mediated transcription, we next used an inactive and a dominantly active mutant of Akt to directly examine the involvement of Akt in LY294002-induced AR repression. Transient transfection assays were performed in LNCaP cells. In the presence of 10 nM DHT, the overexpression of AR induces approximately a 10-fold induction of the PSA promoters. Cotransfection with the wild type β -catenin expression vector augments AR activity to nearly 20-fold above base line (Fig. 4A). The addition of LY294002 to the cells results in a large reduction in AR activity. At 5 μ M LY294002, AR activity was reduced by ~60%. Coexpression of the dominantly active Akt reversed the inhibition of AR activity by LY294002, whereas an inactive mutant of Akt used as a control showed no effect (Fig. 4A). These data directly demonstrate that repression of AR activity by LY294002 is mediated through the down-regulation of PI3K and the subsequent inactivation of Akt activity.

We next performed the transient transfection experiments using either wild type or β -catenin mutants containing a point mutation within the NH₂-terminal GSK3 β binding site. Because these mutants are resistant to GSK3 β -mediated degradation, we further assessed whether the repression of AR by LY294002 is mediated through GSK3 β . As shown in Fig. 4B, an ~40% reduction in expression was induced by 5 μ M LY294002 in the cells that were cotransfected with wild type β -catenin but not in the cells cotransfected with the β -catenin mutants. As mentioned above, because the β -catenin mutants used in these experiments are impervious to the effects of the destructive complex attributed to point mutations within the GSK3 β phosphorylation sites (20), the results from these experiments suggest that GSK3 β is involved in the regulation of β -catenin-mediated augmentation of AR activity.

Expression of PTEN in LNCaP Cells Represses β -Catenin-mediated Augmentation of AR Activity—Recent data have shown that the tumor suppressor PTEN appears to negatively control the PI3K signaling pathway by blocking the activation of the downstream target Akt (24). The mutations in the PTEN gene were found in prostate cancer tissues and cell lines (25). In a previous report, Li *et al.* (28) showed that the transfection of the wild type PTEN repressed an AR-regulated reporter gene in PTEN-null prostate cancer cells. The results from our experiments indicate that the inhibition of PI3K/Akt signaling represses the expression of an endogenous AR target gene and reduces the levels of nuclear β -catenin. To further examine whether repression of AR activity by PTEN is also mediated by PI3K/Akt modulation of nuclear β -catenin, we performed transient transfections using either the wild type β -catenin or the β -catenin mutants described above. As shown in Fig. 5A, in the absence of PTEN vector, both the wild type and β -catenin mutants augment AR-mediated transcription ~1.5-fold using a 7-kilobase PSA promoter in the PTEN-null cells, LNCaP. However, when a wild type PTEN vector was cotransfected into the cells, the wild type β -catenin showed less enhancement of AR

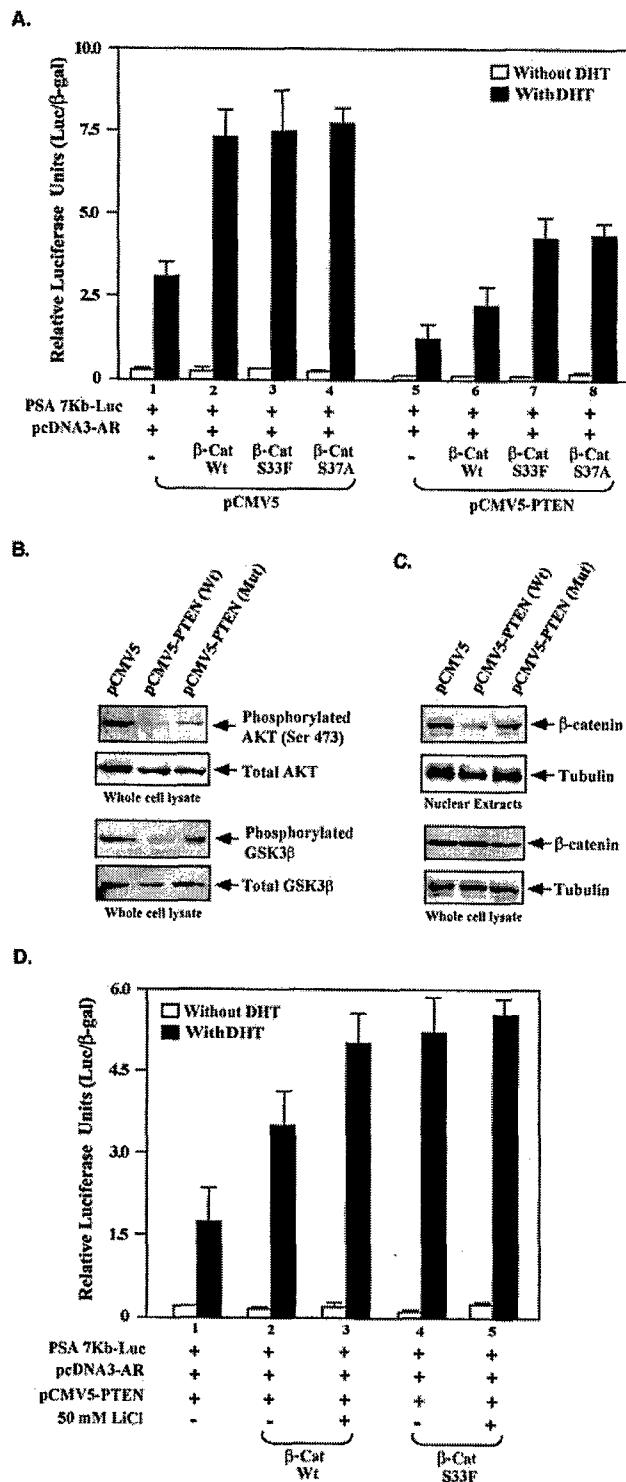


FIG. 5. PTEN represses β-catenin-mediated augmentation of AR activity by reducing nuclear β-catenin protein. *A*, LNCaP cells were transfected with a PSA7kb-luc reporter (100 ng), pcDNA3-β-galactosidase (25 ng), pcDNA3-AR (5 ng), and the wild type or mutants of pcDNA3-FLAG-β-catenin (50 ng) as indicated. Either an empty pCMV5 vector or pCMV5-PTEN was cotransfected with the above plasmids. Ten hours after transfection, the cells were treated with 10 nM DHT or with vehicle only for 18 h. Cell lysates were measured for luciferase and β-galactosidase activities. The data represent the mean \pm S.D. of three independent samples. (*B* and *C*) The PTEN expression constructs were transfected into LNCaP cells. Nuclear extracts and whole cell lysates were prepared from the cells 30 h after transfection and analyzed by Western blotting. *D*, transient transfections were performed with the plasmids as labeled in

activity than the mutants, indicating a repressive effect of PTEN on wild type β-catenin ($p < 0.05$). The results with the mutants of β-catenin demonstrate that the effect of PTEN on AR-mediated transcription is regulated through GSK3β via degradation of nuclear β-catenin. To further confirm this finding, we examined the phosphorylation status of Akt and GSK3β proteins as well as the levels of nuclear β-catenin protein in LNCaP cells, which were transfected with either wild type or the loss-of-function PTEN expression vector. As shown in Fig. 5*B*, both the phosphorylation of Akt and GSK3β proteins was significantly reduced in the cells transfected with wild type PTEN vector. Moreover, a reduction of nuclear β-catenin protein was observed only in the nuclear extracts isolated from cells transfected with the wild type PTEN vector, although the total β-catenin protein detected was almost equal in all of the samples (Fig. 5*C*).

We next examined whether the inhibition of GSK3β can directly affect β-catenin-mediated augmentation of AR activity. As lithium chloride has been shown to inhibit GSK3β through a mechanism independent of serine 9 phosphorylation (37), we examined whether β-catenin-mediated AR augmentation is affected in cells treated with LiCl. As shown in Fig. 5*D*, in the presence of PTEN, the transfection of wild type β-catenin showed less stimulation of AR-mediated PSA promoter activity than that of the mutant β-catenin (black bars 2 and 4). However, the inhibition of GSK3β by LiCl treatment increases AR activity in the presence of wild type β-catenin (black bar 3), whereas there is little change in the PSA promoter activity in the mutant β-catenin-transfected cells treated with LiCl (black bar 5). These data are consistent with previous reports on other human cell lines (36, 38). Taken together, our results demonstrate that PTEN negatively regulates the augmentation of AR activity by β-catenin through targeting of the β-catenin degradation pathway mediated by GSK3β.

DISCUSSION

The PI3K/Akt pathway plays a critical role in prostate cell proliferation and survival (24). PTEN, which is frequently mutated in prostate cancer cells, negatively regulates this process by blocking the PI3K/Akt pathway. Recently, several lines of evidence showed that PI3K/Akt and PTEN can modulate androgen-induced cell growth and AR-mediated transcription in prostate cancer cells (28, 29), suggesting a potential link between the PI3K/Akt and androgen pathways. In this study, we demonstrated that β-catenin acts as the point of convergence for the cross-talk between the PI3K/Akt and androgen signaling pathways. The data presented here are consistent with what is known regarding the degradation of β-catenin by GSK3β, a downstream effector of PI3K/Akt, and fit very well with our recent finding that β-catenin interacts with AR and augments its ligand-dependent transcription (23).

The dysregulation of β-catenin expression and Wnt-mediated signaling is now recognized as important events in the pathogenesis of variety of human malignancies including prostate cancer (18, 39). Tumor cells contain high levels of free cellular β-catenin by acquiring loss-of-function mutations in the components of the destruction complex or by altering regulatory sequences in β-catenin itself. Besides Wnt signaling, other signaling pathways are also involved in regulating cellular β-catenin levels (36, 38, 40). In this study, we showed that PI3K/Akt increases the stability of nuclear β-catenin by phosphorylation and inactivation of the downstream substrate

the figure. After a 10 h transfection, 10 nM DHT and 50 mM LiCl were added to the cells. Whole cell lysates were prepared after another 18 h of incubation and were used to measure luciferase and β-galactosidase activities.

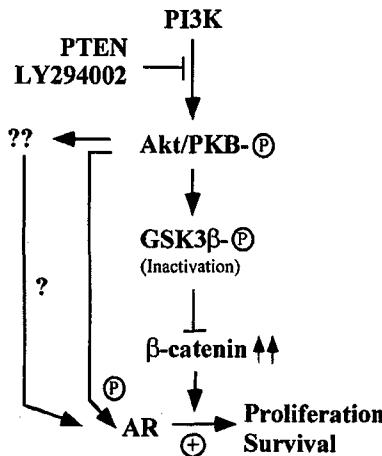


FIG. 6. β -catenin acts as a mediator in the cross-talk between PI3K and androgen signaling. A model summarizes PI3K/Akt signaling in prostate cells and the pathways for PTEN and the PI3K inhibitor LY294002 in the regulation of AR activity.

GSK3 β in prostate cancer cells. Given that β -catenin acts as a transcriptional coactivator of AR, these data provide evidence to suggest a new mechanism whereby PI3K/Akt can affect prostate cell proliferation and survival through androgen signaling.

Earlier studies showed that PTEN negatively regulates the PI3K/Akt pathway in prostate cancer cells (28). The expression of PTEN in LNCaP, a PTEN-null prostate cancer cell line, blocks androgen-induced cell growth and AR-mediated transcription. In this study, we demonstrated that the overexpression of PTEN in LNCaP reduces β -catenin-mediated augmentation of AR activity; however, PTEN showed no effect in cells transfected with β -catenin mutants containing a single point mutation within the GSK3 β phosphorylation sites. The results from our biochemical experiments further demonstrated that PTEN reduces the nuclear accumulation of β -catenin proteins in prostate cells. Because the β -catenin mutants used in our experiments are impervious to degradation by the destruction complex, we conclude that the regulation of β -catenin by PTEN is mediated through GSK3 β . Our results are consistent with a recent study showing that nuclear β -catenin protein is constitutively elevated in PTEN null cells, and this elevated expression can be reduced upon the reexpression of PTEN (41). The data presented here also confirm that PTEN negatively regulates the PI3K pathway by inhibiting phosphorylation of Akt. In addition, the experiments using PTEN as a natural PI3K inhibitor are consistent with our data showing the important effects mediated by the synthetic PI3K inhibitor LY294002.

Modification of the AR protein such as by phosphorylation or acetylation has been suggested to be an important mechanism for modulating AR activity in prostate cancer cells (42–44). The putative consensus sequences for Akt phosphorylation were identified in both the transactivation and the ligand binding domains of AR (29). Those authors showed that Akt can directly bind to and phosphorylate AR (29). However, using both biochemical and functional approaches, we were not able to show a physical protein-protein interaction between Akt and AR or the phosphorylation of AR by Akt *in vitro* (data not shown). Results similar to ours were also reported by Li *et al.* (28). These conflicting results may be attributed to the use of different reagents and experimental conditions, but they also suggest that other alternative pathways may be involved in this regulation (Fig. 6). As presented in this study, we propose a novel molecular mechanism for PI3K/Akt and PTEN regulation of androgen signaling in prostate cancer cells.

The major role of β -catenin in tumorigenesis has been implicated via its interaction with the Tcf/LEF transcription factors (45). Interestingly, as we and others have reported recently (23, 46), β -catenin is shown to have no effect on the activation of Tcf/LEF-mediated transcription in prostate cancer cells despite the expression of Tcf/LEF. A similar observation was also reported recently in breast cancer cells (47). In this study, using Tcf/LEF reporters, we were also not able to demonstrate an effect of PTEN on the regulation by β -catenin of Tcf/LEF-mediated transcription in LNCaP cells (data not shown). This raises the question as to whether the growth-promoting effect of β -catenin is mediated through partners outside of the Tcf/LEF pathway in prostate cancer and/or other tumor cells.

In this study, we demonstrate that β -catenin mediates the cross-talk between PI3K/Akt and androgen pathways. Based on these results and previous studies by others, we summarize our findings in Fig. 6. The PI3K/Akt signal induces phosphorylation and inactivation of GSK3 β , resulting in increased nuclear levels of β -catenin. Consequently, increased β -catenin elevates AR activity to stimulate prostate cell growth and survival. Both the PI3K inhibitor LY294002 and PTEN negatively regulate these processes. A loss-of-expression or mutational inactivation of PTEN has been frequently observed in human tumors, which induce the suppression of apoptosis and accelerates cell cycle progression (24, 25). Additionally, the mutation or aberrant expression of the destruction complex and the reduction of E-cadherin, which results in increased nuclear β -catenin, also occurs during prostate cancer progression (39). Our data showing that PTEN reduces nuclear β -catenin in prostate cancer cells suggest a novel role of PTEN in down-regulating androgen-induced cell growth and survival. A further study of the regulation of the interaction among PI3K, Wnt, and the androgen signaling pathways in prostate cancer cells should provide fresh insight into the pathogenesis of prostate cancer that may help us to identify new pathways that can be targeted for prostate cancer treatment.

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hZimp10 is an androgen receptor co-activator and forms a complex with SUMO-1 at replication foci

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The androgen receptor (AR) plays a central role in male sexual development and in normal and malignant prostate cell growth and survival. It has been shown that transcriptional activation of AR is regulated through interaction with various co-factors. Here we identify a novel PIAS-like protein, hZimp10, as an AR-interacting protein. The transactivation domain (TAD) of AR and the central region of hZimp10 were found to be responsible for the interaction. A strong intrinsic transactivation domain was identified in the C-terminal, proline-rich region of hZimp10. Endogenous AR and hZimp10 proteins were co-stained in the nuclei of prostate epithelial cells from human tissue samples. In human prostate cancer cells, hZimp10 augmented the transcriptional activity of AR. Moreover, hZimp10 co-localized with AR and SUMO-1 at replication foci throughout S phase, and it was capable of enhancing sumoylation of AR *in vivo*. Studies using sumoylation deficient AR mutants suggested that the augmentation of AR activity by hZimp10 is dependent on the sumoylation of the receptor. Taken together, these data demonstrate that hZimp10 is a novel AR co-regulator.

Keywords: androgen receptor/nuclear hormone receptors/PIAS/prostate cancer/sumoylation

Introduction

The effects of androgens are mediated by the androgen receptor (AR), which plays a critical role in male sexual development and in prostate cell growth and survival (Jenster, 1999). AR belongs to the nuclear receptor superfamily and contains four functional domains: a transactivation domain (TAD), a DNA-binding domain (DBD), a hinge region and a ligand-binding domain (LBD) (Zhou *et al.*, 1994). The unbound AR forms a complex with heat-shock proteins (HSPs) (Sanchez *et al.*, 1990). Upon binding to ligand, the AR dissociates from the HSPs and translocates into the nucleus, where it binds to the

androgen response element (ARE) and stimulates ligand-dependent transcription (Zhou *et al.*, 1994). Like other receptors, AR can bind to different co-factors through its distinct functional domains (Heinlein and Chang, 2002). Through such interactions, the co-factors can modulate AR activity. One of the mechanisms by which co-factors regulate AR activity is by modification of the AR protein. It has been documented that the AR can be modified by phosphorylation (Nazareth and Weigel, 1996), acetylation (Fu *et al.*, 2000) and sumoylation (Poukka *et al.*, 2000) in cells.

Members of the PIAS (protein inhibitor of activated STAT) family were originally identified as transcriptional co-regulators of the JAK–STAT pathway (Chung *et al.*, 1997). PIAS1 and PIAS3 have been shown to block DNA binding of STAT1 and STAT3, respectively, and to inhibit their action (Chung *et al.*, 1997; Liu *et al.*, 1998). Crosstalk between PIAS proteins and other signaling pathways has also been demonstrated to be involved in various cellular processes (see review by Jackson, 2001). PIAS α was first isolated as an AR-interacting protein (ARIP3), which binds to AR and modulates AR-mediated transcription (Moilanen *et al.*, 1999). Other members of the PIAS family have also been shown to play a role in regulating the activity of AR and other nuclear hormone receptors (Kotaja *et al.*, 2000; Tan *et al.*, 2002).

PIAS and PIAS-like proteins share a zinc finger domain, termed Miz, in the central region (Wu *et al.*, 1997). The Miz domain (Msx-interacting zinc finger) was shown to mediate the interaction between Msx2 and PIAS β . An increasing number of proteins from invertebrates have been found to contain the Miz domain, suggesting a conserved and biologically important role of PIAS proteins throughout evolution.

The SUMO (small ubiquitin-related modifier) conjugation system has been extensively studied and it shares similarity with the ubiquitin-conjugation system (Melchior, 2000). Modification by SUMO-1 (sumoylation) is a three-step process, involving the E1 enzyme Aos1/Uba2, the E2 enzyme Ubc9 and the recently identified E3-like ligases, such as the nucleoporin RanBP2 (Melchior, 2000; Jackson, 2001; Pichler *et al.*, 2002). While modification of proteins by SUMO-1 is a covalent process, it is reversible through the activity of a number of specific isopeptidase enzymes (Nishida *et al.*, 2001). Although the specific mechanism by which SUMO-1 modification modulates cellular functions remains unclear, it is believed that unlike ubiquitination, sumoylation does not promote protein degradation but rather is involved in mediating protein–protein interactions, subcellular compartmentalization and protein stability. Recent data suggest that sumoylation is important in the regulation of transcription (Verger *et al.*, 2003). Sumoylation of both steroid

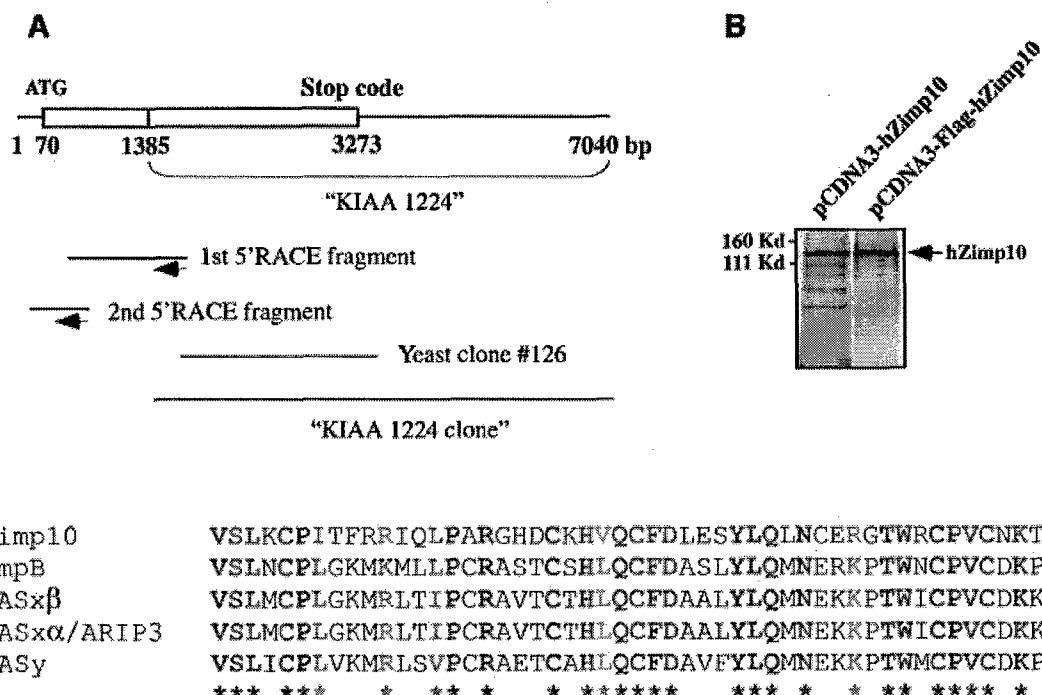


Fig. 1. Nucleotide and predicted amino acid sequence of hZimp10. **(A)** Diagram of the hZimp10 transcript. Both 5' and 3' untranslated regions are shown as a solid line and the coding region is displayed as an open box. The translation start site (ATG) and stop codon are labeled with nucleotide numbers. **(B)** The pcDNA3 constructs containing full-length hZimp10 cDNA, either with or without Flag-epitope tag, were transcribed and translated *in vitro*. Proteins were labeled and separated on an SDS-PAGE. **(C)** The full-length cDNA and predicted amino acid sequences of hZimp10 are shown (DDBJ/EMBL/GenBank accession No. AY235683). The putative functional domains are colored: a nuclear localization signal (green), a Miz domain (red) and two proline-rich regions (pink). **(D)** Alignment of the Miz domain of hZimp10 with those of other PIAS proteins is shown. Identical or similar amino acids are shaded with either blue or orange.

receptors and their co-factors have been demonstrated (Poukka *et al.*, 2000; Kotaja *et al.*, 2002).

Recently, PIASx α , x β , 1 and 3 have been found to interact with SUMO-1 and Ubc9 and mediate sumoylation of steroid receptors and other transcription factors (Kahyo *et al.*, 2001; Kotaja *et al.*, 2002; Nishida and Yasuda, 2002). PIAS proteins are also modified by sumoylation and are involved in the process of sumoylation of other binding partners (Jackson, 2001; Kotaja *et al.*, 2002). Here we identify a novel AR-interacting protein that augments AR-mediated, ligand-dependent transactivation in prostate cells. Sequence analysis of the protein showed that it contains a conserved Miz domain and is a new PIAS-like protein. Based on these features, we named the protein as hZimp10 (human Zinc finger-containing, Miz1, PIAS-like protein on chromosome 10). Specific interaction between AR and hZimp10 was demonstrated both *in vitro* and *in vivo*. Importantly, endogenous AR and hZimp10 proteins were co-stained in the nuclei of prostate epithelial cells in human tissue samples. Fusion of hZimp10 to a heterologous DNA-binding domain revealed a strong transactivation domain in the C-terminal region of hZimp10. In addition, we have demonstrated that hZimp10 co-localizes with AR and SUMO-1 in the nucleus and forms a protein complex at replication foci. Moreover, we showed that hZimp10 is able to enhance the sumoylation of AR in intact cells. Using AR with mutations in the two major sumoylation sites, we

demonstrated that augmentation of AR activity by hZimp10 is dependent on the sumoylation of the receptor. Thus, these data demonstrate that hZimp10 acts as an AR co-regulator and modifies AR activity in transcription and DNA replication.

Results

Isolation of a novel PIAS-like protein, hZimp10

To understand the mechanism of AR-mediated trans-activation of genes, we employed a modified yeast two-hybrid system using a bait construct containing a partial TAD sequence [amino acids (aa) 1–333] of the AR (James *et al.*, 1996). Of 7.4×10^6 transformants, 13 grew under selective conditions and showed increased β -galactosidase (β -gal) production. Rescue of the plasmids and sequencing of the inserts revealed several different cDNAs. Among them, two cDNAs (clone numbers P126 and P233) encoded a novel sequence, and analysis of these mouse cDNA clones with the NCBI DDBJ/EMBL/GenBank database suggested that the human KIAA1224 cDNA clone was the homolog. Since KIAA1224 is a truncated fragment, we performed the rapid amplification of 5' cDNA ends (5'-RACE) to isolate the full-length cDNA (Figure 1A). Sequence analysis of the full-length clone created by combining the 5'-RACE fragments and the KIAA1224 clone revealed a methionine initiation codon at nucleotide 70, followed by an open reading frame

encoding a 1067 aa protein with a predicted molecular weight of 123 kDa (DDBJ/EMBL/GenBank accession No. AY235683) (Figure 1C). Using *in vitro* transcription and translation, an ~130 kDa protein was generated by the full-length clone (Figure 1B), confirming the identity of the predicted initiation codon. The clone with an N-terminal Flag epitope-tag encoded a protein with a molecular weight similar to the clone with the natural initiation codon.

A BLAST search of the human genome database showed that this full-length sequence is located on human chromosome 10 at 10q22.1–22.3 and is comprised of 21 putative exons. Analysis of the protein sequence showed that the clone contains several functional domains, including a Miz domain, a nuclear localization signal sequence and two proline-rich regions (Figure 1C). PIAS and PIAS-like proteins share a highly conserved Miz domain, and a high degree of sequence similarity was observed when our clone was aligned with the zinc fingers of other PIAS proteins (Figure 1D). Based on these features, we named this protein as hZimp10 (human zinc finger-containing, Miz1, PIAS-like protein on chromosome 10).

hZimp10 interacts with the AR

We initially identified Zimp10 through a yeast two-hybrid screen with a 'bait' containing the partial TAD of AR. To confirm the interaction and identify the specific region of AR responsible for the interaction, we co-transformed either the clone of Zimp10 that was isolated from the initial screen or the constructs containing either GAL4 DBD alone or various fusions of different fragments of AR, respectively (Figure 2A). A liquid β -gal assay was performed to quantify the interactions. The AR/TAD-DBD and AR/pTAD1 constructs showed an ~23- or 16-fold induction with pVP16-Zimp10 compared with pVP16 alone. However, the clone with deletion of the region between aa 243–333 (AR/pTAD2) showed virtually no interaction with Zimp10, suggesting that the region between aa 243–333 is critical for the interaction. No significant production of β -gal was observed in the samples co-transformed with Zimp10 and other AR constructs containing the DBD, DBD-LBD and LBD.

To map the interaction region of Zimp10, we generated several truncated mutants of hZimp10 and assessed their abilities to interact with AR. As shown in Figure 2B, the full-length fragment and the C-terminal deletion mutant containing aa 1–790 possess strong interaction activity. Further deleting the C-terminal sequence of hZimp10 gradually reduces β -gal activity. The fragment containing aa 1–333 showed only minimal activity. The mutant containing aa 556–1067 showed the highest levels of β -gal activity among the N-terminal truncation mutants. These results suggest that the region between aa 556 and 790 contributes to the interaction with the AR. An additional mutant that contains only the central region of the protein (aa 556–790) was generated and used to map the precise interaction region. As shown in Figure 2B, this mutant showed the highest β -gal activity, indicating that the central region between aa 556 and 790 is the primary binding region for AR.

To confirm that AR and hZimp10 are physically associated in intact cells, co-immunoprecipitation assays

were carried out to detect a possible protein complex. We expressed the Flag-tagged hZimp10 together with AR in CV-1 cells. Whole-cell lysates containing equal amounts of proteins were immunoprecipitated with normal mouse IgG or an anti-Flag monoclonal antibody. As shown in Figure 2C, Flag-hZimp10 proteins were detected in the immunoprecipitates with anti-Flag antibody, but not in the ones with normal IgG. These data demonstrate that an interaction between AR and hZimp10 occurs in mammalian cells.

hZimp10 is selectively expressed in human ovary, testis and prostate

Northern blot analysis was carried out to examine the expression of hZimp10. Three probes isolated from the N-terminal (aa 1–196), central (aa 330–496) and C-terminal (aa 880–1060) regions of the hZimp10 cDNA were used to ensure that the full-length sequence of hZimp10 constructed by combining three cDNA fragments together corresponded to a natural transcript (Figure 3A). A 7.2 kb hZimp10 transcript was detected by all three probes in various tumor cell lines (Figure 3B), which is consistent with the cDNA size of hZimp10 (Figure 1C). LAPC4, LNCaP, MCF7 and PC3 showed relatively high signals (Figure 3B and C). Using probe 1, we also examined the transcript profile of hZimp10 in human tissues. As shown in Figure 3D, a 7.2 kb transcript of hZimp10 was detected most abundantly in ovary, and at lower levels in prostate, spleen and testis. There was little or no detectable signal in thymus, small intestine, colon and peripheral blood leukocytes.

hZimp10 contains a very strong transactivation domain

Next, we investigated a possible role for hZimp10 in transcription. Fragments containing the full-length or N-terminal truncation mutants of hZimp10 were targeted to DNA by fusion with the GAL4 DBD. These constructs were then tested for their abilities to modulate transcription from a minimal promoter, derived from the chicken myelomonocytic growth factor gene (Sterneck *et al.*, 1992). Fusion of the GAL4 DBD to the full-length fragment of hZimp10 showed an ~7-fold induction compared with the GAL4 DBD alone (Figure 4). Deletions of the N-terminal region between aa 1 and 556 did not significantly affect transcription. However, removal of the region between aa 556 and 692 produced a dramatically elevated transcriptional activity. The mutant containing the C-terminal proline-rich region (aa 829–1067) showed 60- to 80-fold more transcriptional activity than that of the full-length hZimp10 construct. It should be noted that the transcriptional activity mediated by the C-terminal proline-rich region of hZimp10 is much stronger than that mediated by the TADs of other transcriptional factors that we have examined, including p53, Smad3, PU1, GATA2, AR and estrogen receptor α (ER α) (data not shown), and is comparable to the activity of the TAD of VP16 (Figure 4). Identification of the strong transcriptional activation domain confirms a functional role for hZimp10 in transcriptional regulation, providing the first line of evidence showing that a PIAS-like protein can regulate transcription through an intrinsic TAD.

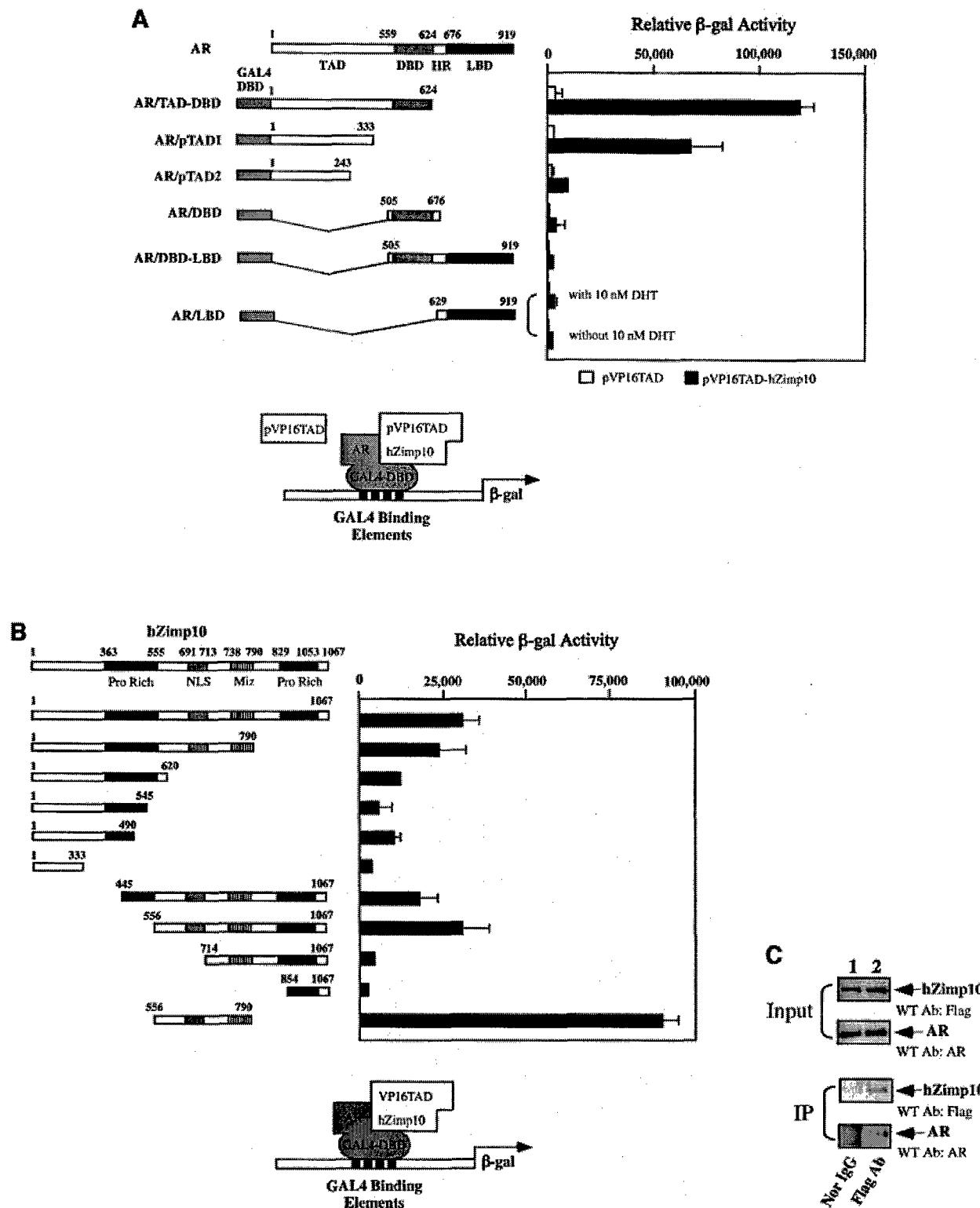


Fig. 2. Specific interaction between hZimp10 and AR. (A) cDNA fragments containing different portions of the human AR were fused to GAL4DBD in the pGBT9 vector. Numbers correspond to amino acid residues. The AR vectors were co-transformed with the pVP16 vector alone or the pVP16-Zimp10 construct. Three independent colonies were inoculated from each transformation experiment for a liquid β -gal assay. The data are shown as the mean \pm SD. (B) Different truncation mutants of hZimp10 were generated by fusing fragments of hZimp10 to the TAD of VP16, and co-transformed with the pGBT9-AR/pTAD1. Transformants were selected and analyzed. (C) CV-1 cells were transiently co-transfected with AR and Flag-tagged hZimp10. Equal amounts of whole-cell lysates were blotted with AR or Flag antibodies to detect expression of the two proteins (input) and subjected to immunoprecipitation with normal mouse IgG or anti-Flag monoclonal antibody. The precipitated fractions were then resolved by SDS-PAGE and analyzed by western blotting using anti-Flag antibody or anti-AR antibody (IP).

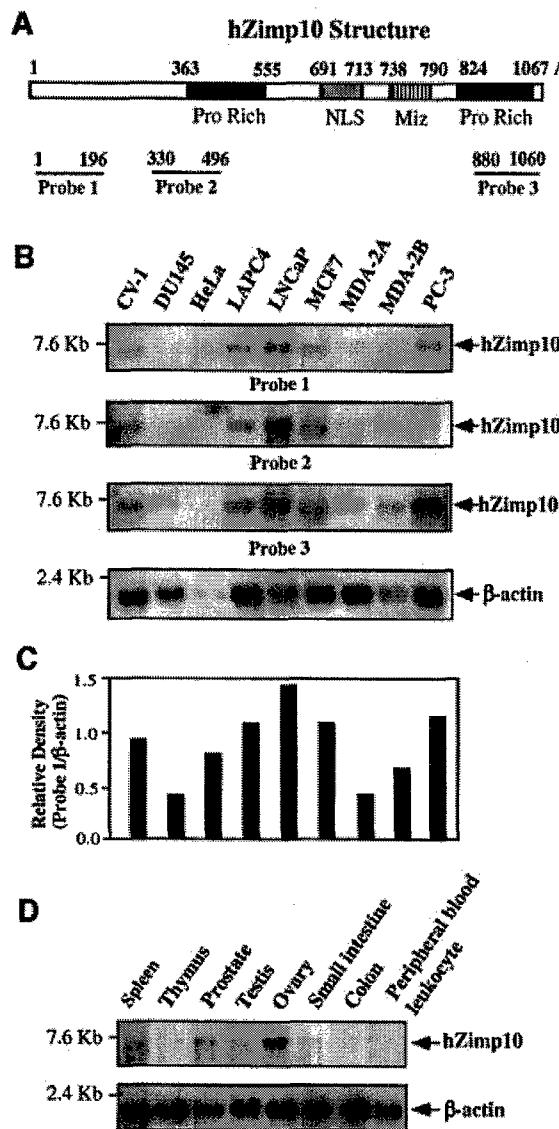


Fig. 3. Expression of hZimp10 in cell lines and human tissues. (A) A schematic representation of the hZimp10 protein is shown with the locations of three probes indicated. The numbers correspond to the amino acid sequence of the protein. (B) Northern blots containing poly(A)⁺ RNA samples from various cell lines were hybridized with the three hZimp10 probes and a probe derived from human β -actin cDNA. (C) Relative densities (signals of probe 1 divided by those of β -actin) were used to measure the expression levels. (D) The blot with multiple human tissues was probed with probe 1 and the fragment of β -actin.

hZimp10 selectively augments AR-mediated transcription

Given the identification of an intrinsic activation domain within hZimp10 and a protein–protein interaction between AR and hZimp10, we further examined the effects of hZimp10 on AR-mediated transcription. Transient transfection experiments were carried out with plasmids expressing AR, hZimp10, and a luciferase reporter driven by the 7 kb promoter of the prostate-specific antigen (PSA) gene (Pang *et al.*, 1997). A basic ligand-dependent transactivation was observed in the CV-1 cells transfected with AR plasmid alone (Figure 5A). Co-transfection of

hZimp10 construct augmented AR activity. With 20 ng of hZimp10 plasmid, AR-dependent transcription was increased 4-fold. To ensure that augmentation of the PSA promoter by hZimp10 was mediated through the AR rather than through other transcription factors, we examined the effect of hZimp10 on the transcription from a luciferase reporter driven by a minimal promoter with two AREs. A similar ligand-dependent enhancement of AR-mediated transcription by hZimp10 plasmid was observed from the ARE-luciferase reporter (Figure 5B). These results indicate that hZimp10 functions as a co-activator of AR and is able to enhance AR-mediated transcription.

To evaluate the enhancement by hZimp10 in a physiologically more relevant cellular context, an AR-positive prostate cancer line, LNCaP, was transfected with an hZimp10 construct along with the luciferase reporter driven by the PSA promoter. As shown in Figure 5C, overexpressed hZimp10 enhanced endogenous AR-mediated transcription from the PSA promoter. These data demonstrate the capability of hZimp10 to augment endogenous AR activity in prostate cancer cells.

The specificity of hZimp10-mediated augmentation was further investigated with other nuclear receptors, including the glucocorticoid receptor (GR), progesterone receptor (PR), ER α , thyroid receptor β (TR β), and vitamin D receptor (VDR). As shown in Figure 5D, there was no significant effect of hZimp10 on GR, PR, ER α and VDR-mediated transcription. hZimp10 showed a slight enhancement of TR β -mediated transcription. These results are consistent with our previous observation that there is no significant interaction between the hZimp10 protein and other nuclear receptors in yeast (data not shown).

hZimp10 protein is specifically expressed in prostate epithelial cells and co-stained with the AR protein

Our results demonstrate that hZimp10 is a novel AR-interacting protein that augments AR-mediated transcription. To further explore the potential biological role of hZimp10, we examined the expression of hZimp10 in human prostate tissues. Two adjacent sections from each tissue sample were stained with either anti-AR or anti-hZimp10 antibody. As reported previously, AR was found exclusively in the nucleus of prostate epithelial cells (Figure 6A, C, E and G). hZimp10 proteins showed a strong nuclear and cytoplasmic staining in prostate epithelial cells (Figure 6B, D, F and H). There was no, or very weak, staining in the stromal elements with either antibody in all samples examined. Comparing the staining patterns from the two adjacent sections, a clear co-staining of AR and hZimp10 proteins was found in the nucleus of prostate epithelial cells. These data provide the first line of evidence that hZimp10 can interact with AR in a biologically relevant manner and indicate that hZimp10 may play a role in modulating androgen signaling.

hZimp10 is found at cell cycle-regulated DNA replication foci throughout S phase

A number of PIAS proteins have been shown to localize to the nucleus and to display speckled patterns of nuclear distribution (Kotaja *et al.*, 2002). Transfection with expression plasmids encoding Flag-tagged hZimp10 in CV-1 cells showed a similar pattern of nuclear distribution

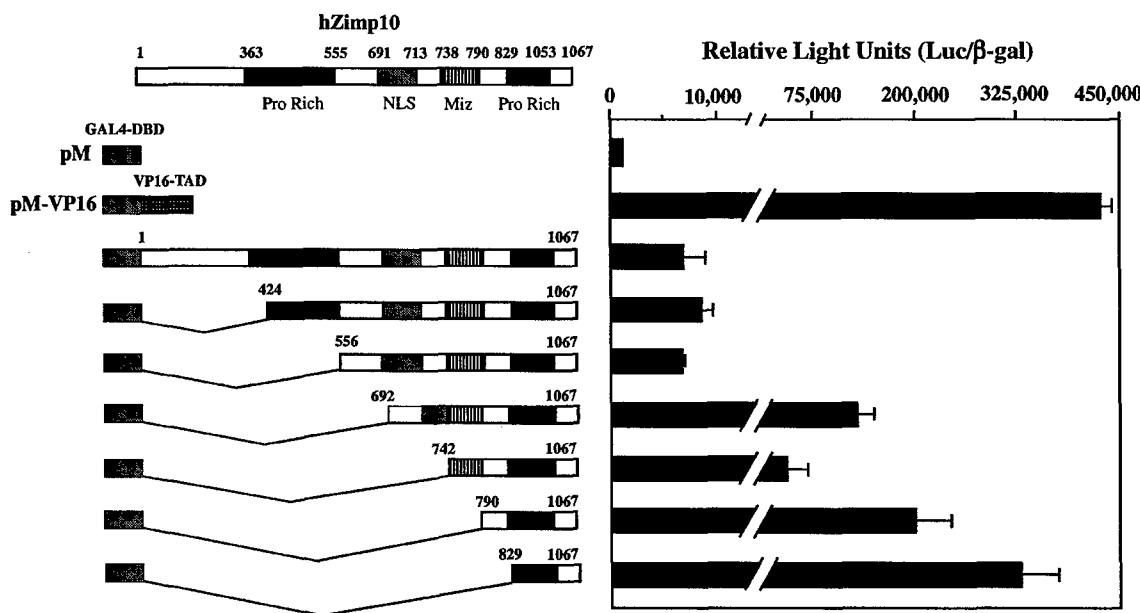


Fig. 4. Detection of intrinsic transcriptional activity of hZimp10. Full-length or truncated fragments of hZimp10 were fused to GAL4 DBD in the pM vector. Numbers correspond to amino acid residues. The pM constructs were co-transfected with luciferase reporter constructs containing the chicken myelomonocytic growth factor gene minimal promoter (-41 to +61) into CV-1 cells. Data are presented in relative light units (RLU), which were obtained by normalizing the activities of luciferase to those of β -gal. The results are reported as the mean \pm SD from representative experiments.

as with the Flag-ARIP3 plasmid (data not shown), suggesting that hZimp10 may be involved in DNA replication structures in interphase nuclei. Distinct patterns of DNA replication have been identified during S phase in mammalian cells, and the size, shape and number of replication foci change during cell cycle progression (O'Keefe *et al.*, 1992). Using immunofluorescence imaging, we systematically probed a potential role of hZimp10 in DNA replication. Cells were synchronized in late G₁ phase with mimosine and then allowed to enter S phase (Krude, 1999). Newly synthesized DNA was identified by 5-bromodeoxyuridine (BrdU) labeling and staining. An identical pattern of replication foci in synchronized cells was observed during S phase progression (Figure 7, left panel). Replication foci changed from numerous small, punctate structures in early S phase cells to large, toroidal structures in late S phase cells. Intriguingly, hZimp10 labeled by the Flag monoclonal antibody and newly synthesized DNA labeled by BrdU displayed a similar pattern of nuclear distribution (Figure 7, left and middle panels), and were overlaid throughout S phase (right panel). Our results demonstrate that hZimp10 can be found at sites of DNA synthesis throughout all phases of DNA replication, suggesting that hZimp10 may have a role in DNA synthesis.

hZimp10 co-localizes with AR and SUMO-1 during cell cycle progression

Next, we examined whether hZimp10 co-localizes with AR during cell cycle progression. To more systematically characterize localization of AR and hZimp10 throughout S phase, we synchronized cells in late G₁ phase, and then allowed the cells to progress through S phase. In cells synchronized in late G₁ phase, both AR and Flag-hZimp10 showed diffuse nuclear staining (Figure 8A). When

merged, these staining patterns showed a considerable amount of overlaying (yellow) throughout the nucleus. When the cells were allowed to progress into S phase, Flag-hZimp10 became associated with the distinctive small punctate structures of early S phase replication foci, whereas a portion of AR tended to retain a diffuse nuclear staining pattern (Figure 8B). When the cells progress further into S phase, Flag-hZimp10 demonstrated the slightly larger punctate staining indicative of mid-S phase (Figure 8C–E), and then the large, toroidal replication foci characteristic of late S phase (Figure 8F). A significant amount of overlaying between Flag-hZimp10 and AR appeared throughout S phase, although a portion of AR remained diffusely localized throughout the nuclei. Based on these observations, we conclude that hZimp10 can co-localize with AR in replication foci throughout S phase and that in addition to its role in transcription, AR may play a role in DNA replication.

Previous studies have shown that PIAS proteins can interact with SUMO-1 and function as SUMO-1 ligases (Kotaja *et al.*, 2002). To further examine whether hZimp10 forms a protein complex with SUMO-1, we repeated the above experiments with cells co-transfected with both green fluorescent protein (GFP)–SUMO-1 and Flag-hZimp10. CV-1 cells were synchronized in late G₁ phase. As shown in Figure 9A, both GFP–SUMO-1 and Flag-hZimp10 displayed a diffuse pattern of nuclear staining in late G₁. When cells progressed into S phase, both of the proteins were found in the small, punctate structures of replication foci in early S phase (Figure 9B and C) and in the large, toroidal structures in late S phase (Figure 9D–F). To confirm that SUMO-1 localizes at replication foci, we labeled newly synthesized DNA with BrdU in the cells transfected with the GFP–SUMO-1 plasmid. As shown in Figure 10, the staining of SUMO-1

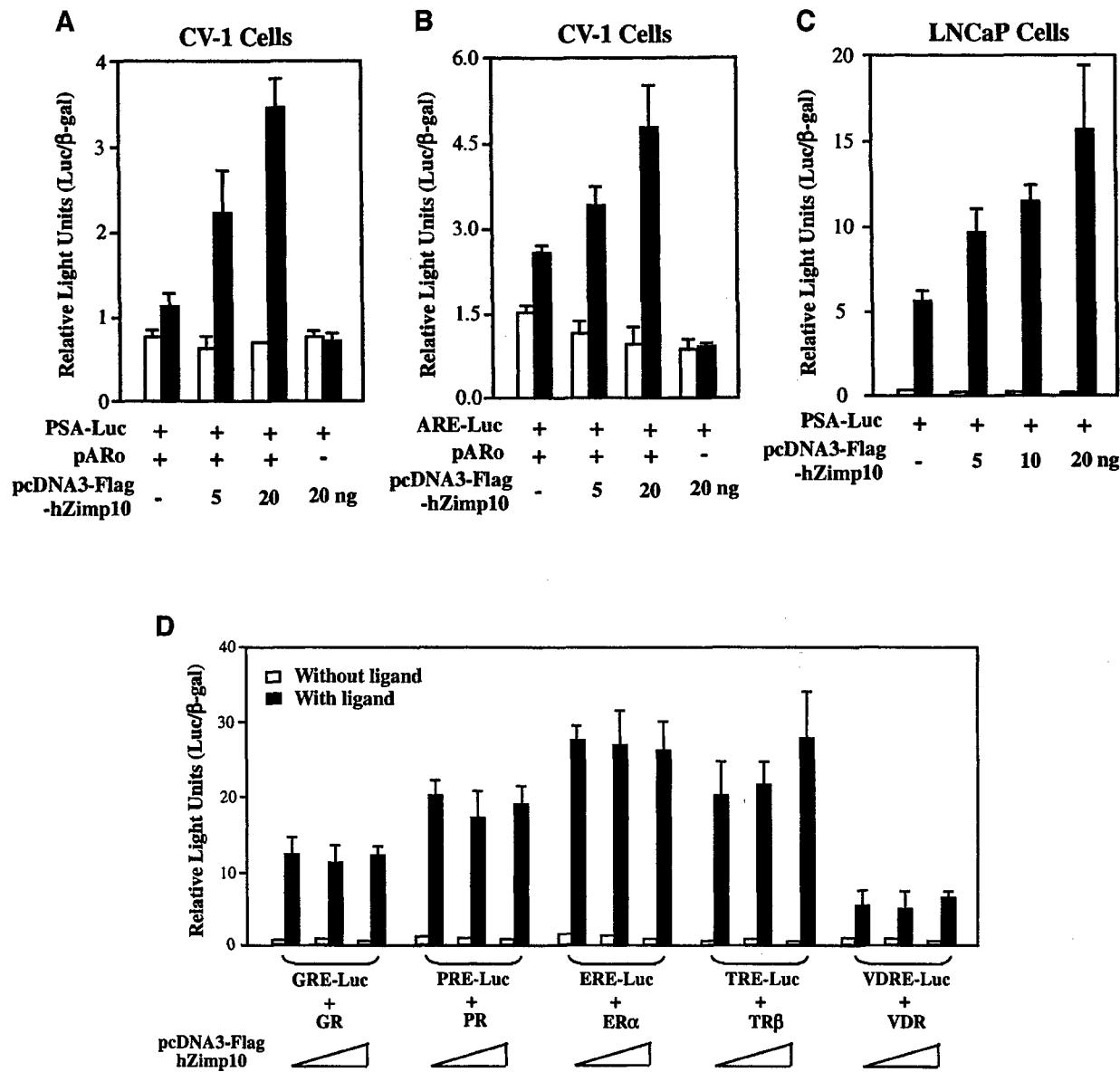


Fig. 5. hZimp10 selectively enhances AR-mediated transcription. (A) CV-1 cells were transfected with a luciferase reporter driven by the 7 kb PSA promoter (100 ng), pcDNA3-β-gal (25 ng), pSV-hARO (5 ng) and different amounts of the pcDNA3-Flag-hZimp10 as indicated. Twenty-four hours after transfection, cells were incubated with or without 10 nM DHT for 24 h. Cell lysates were prepared for assessment of luciferase and β-gal activities. (B) Similar to (A), except that a 2×ARE-luc reporter (100 ng) was used. (C) LNCaP cells were transfected with the PSA7kb-luc reporter (100 ng), pcDNA3-β-gal (25 ng) and the pcDNA3-Flag-hZimp10 as indicated. Twenty-four hours after transfection, cells were treated with or without 10 nM DHT for 24 h. Cell lysates were measured for luciferase and β-gal activities as described above. (D) One hundred nanograms of luciferase reporters driven by different response elements, as labeled in the figure, were co-transfected with 50 ng of pSV40-β-gal and 10 ng of different receptors into CV-1 cells. Either 0, 5 or 20 ng of pcDNA3-Flag-hZimp10 was co-transfected. Cells were cultured either in the presence or absence of the specific ligands to each receptor, including 10 nM dexamethasone (DEX), 10 nM progesterone, 100 nM β-estradiol, 10 nM tri-iodothyronine and 10 nM 1α, 25-dihydroxyvitamin D3.

was mainly overlaid with the BrdU staining throughout S phase, particularly in the late time point. The results demonstrate that SUMO-1 can localize at DNA replication foci, suggesting a role of SUMO-1 in DNA synthesis. In addition, we also confirmed the above observations through the co-localization of endogenous hZimp10, SUMO and AR with BrdU during S phase (see Supplementary data, available at *The EMBO Journal* Online).

hZimp10 is able to enhance the sumoylation of AR and the augmentation of hZimp10 is linked to the AR sumoylation sites

It has been shown that members of PIAS family can function as SUMO ligases (Kotaja *et al.*, 2002). To determine whether hZimp10 has a role in the sumoylation of AR, we co-transfected hZimp10, AR and SUMO-1 into COS-1 cells. As shown in Figure 11A, the expression of hZimp10 with AR in the presence of different amounts of

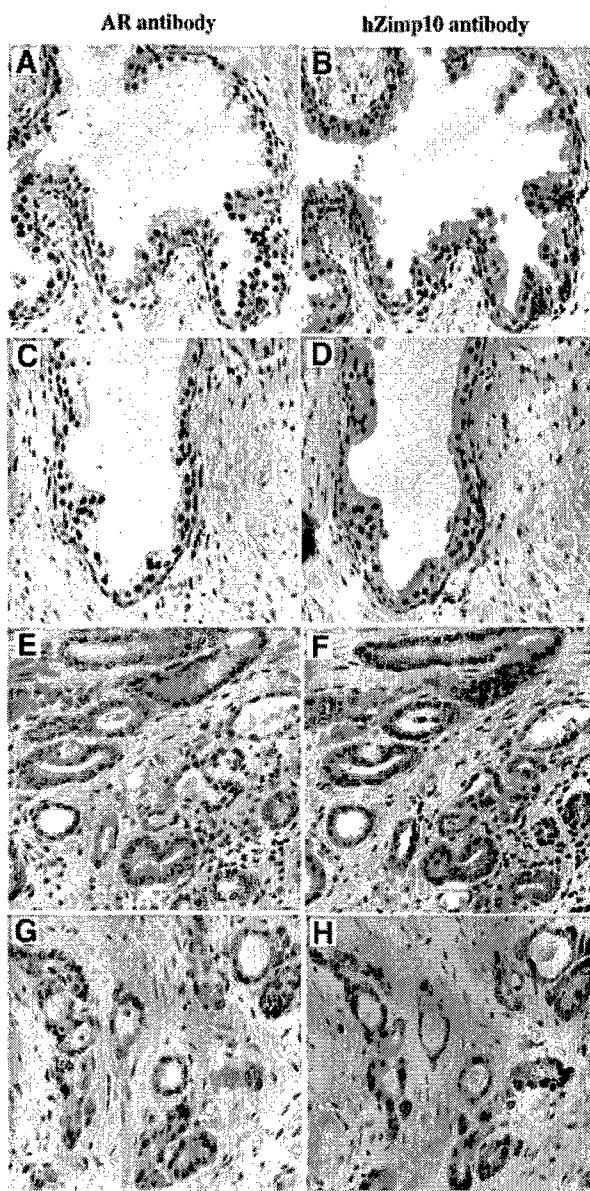


Fig. 6. The AR and hZimp10 co-localize in prostate epithelial cells. Four pairs of human prostate tissue samples (A–H) were stained either with anti-AR (left panel) or anti-hZimp10 (right panel) antibody. Color was developed with DAB in PBS. All sections used for immunohistochemistry were lightly counterstained with 5% (w/v) Harris hematoxylin.

SUMO-1 enhanced the intensity of the two slowly migrating AR immunoreactive bands. Although the effect of hZimp10 was relatively modest in comparison with that of PIAS-1, there was an ~40% increase in the intensity of the sumoylated AR forms in the presence of 0.04 or 0.1 μ g of SUMO-1. Our results indicate that hZimp10 is capable of enhancing the sumoylation of AR.

The two major sumoylation sites (K386 and K520) have been identified within the AR (Poukka *et al.*, 2000). Mutation of these sites significantly reduces the sumoylation of AR. Using constructs containing either single or double mutation at the above amino acids, we studied a link between the sumoylation and transactivation of AR.

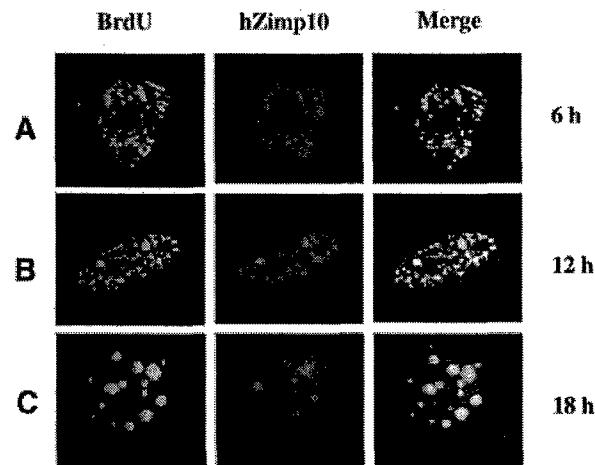


Fig. 7. Immunofluorescent localization of hZimp10 at replication foci. The pcDNA3-Flag-hZimp10 construct was transfected into CV-1 cells. Cells were synchronized with 0.5 mM mimosine (see Materials and methods). Representative confocal laser scanning microscopy images of nuclei from cells expressing Flag-tagged hZimp10 proteins and pulsed with BrdU are shown. Color was developed with either FITC-conjugated monoclonal anti-BrdU antibody (green) or the anti-Flag primary antibody followed by secondary antibodies conjugated with rhodamine (red). Merge (right panel) of left and middle panels indicates areas of co-localization (yellow).

Transfection of the wild-type or mutant AR with a luciferase reporter driven by the 7 kb PSA promoter showed variable ligand-dependent activity (Figure 11B). Mutation at one sumoylation site (K386R) reduced the activity of AR by ~50%, and mutation of both sumoylation sites (K386R/K520R) further decreased the residual activity by another 50%. These results suggest that sumoylation of AR is essential for the activity of AR. The link between sumoylation and transactivation of AR by hZimp10 was further tested by co-transfection of hZimp10 with the wild-type and mutants of AR. As we observed previously, co-transfected hZimp10 enhanced the transcriptional activity of the wild-type AR (Figure 11B). Interestingly, hZimp10 was not able to modulate the activity of the mutant ARs. In contrast, a well-characterized AR co-activator ARA70 (Yeh and Chang, 1996) augmented both the wild-type and the mutants of AR, albeit to a different degree. Taken together, the results suggest that sumoylation of AR is involved in hZimp10-mediated augmentation of AR activity. Thus, given the fact that hZimp10 is able to enhance the sumoylation of AR, we have provided a link between hZimp10-mediated enhancement of AR sumoylation and modulation of AR-mediated transcription.

Discussion

Like other nuclear hormone receptors, transcriptional activity of the AR can be modulated through interactions with various co-regulators (Rosenfeld and Glass, 2001). Aberrations in the expression or function of these co-factors may lead to enhancement of AR activity and provide an adaptive advantage for tumor cell growth. Changes in the transcriptional programs of the AR are important but poorly understood events in prostate cancer

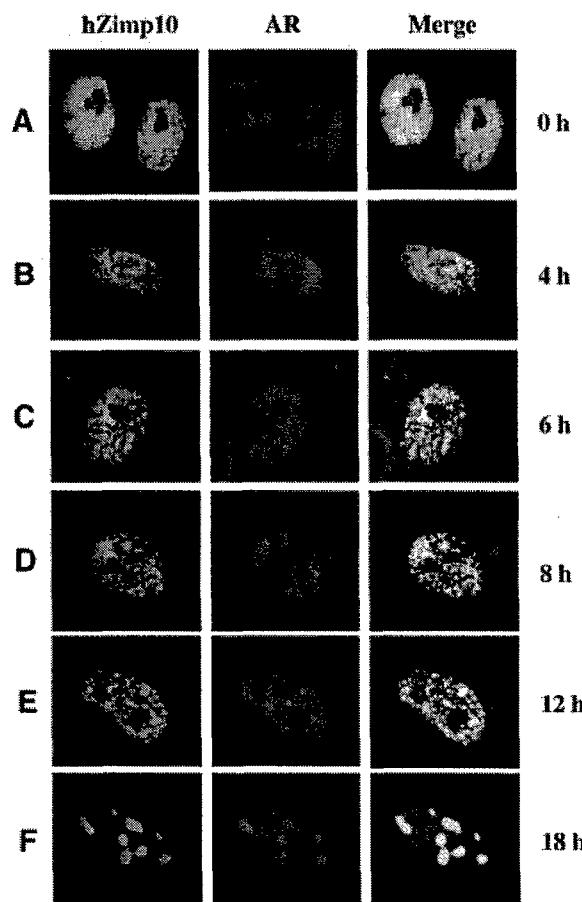


Fig. 8. Immunofluorescent co-localization of hZimp10 and AR. CV-1 cells co-transfected with pcDNA3-Flag-hZimp10 and pSVARo were synchronized with mimosine. Double immunostaining was conducted with anti-Flag and anti-AR antibodies followed by the secondary antibodies conjugated with FITC (green) and rhodamine (red), respectively. Representative confocal laser scanning microscopy images of nuclei from cells expressing Flag-tagged hZimp10 and AR proteins are shown. Merged images demonstrating co-localization of proteins are shown on the right panels (yellow). Staining was performed at different time points, including late G₁ phase before entering S phase (0 h, A), early S phase (4 and 6 h, B and C), mid-S phase (8 and 12 h, D and E) and late stages of S phase (18 h, F).

development and progression. In our search for AR co-regulators that contribute to prostate cancer tumorigenesis, we identified a novel PIAS-like protein, hZimp10, which interacts with AR and augments AR-mediated transcription. Importantly, endogenous AR and hZimp10 are co-stained in the nuclei of prostate epithelial cells in human prostate tissues. This intriguing evidence, combined with other evidence, strongly suggests that the interaction between hZimp10 and AR is biologically relevant and may play a role in prostate cells.

In this study, we tested the functional consequences of the interaction between AR and hZimp10. We demonstrated that hZimp10 augments the ligand-dependent activity of AR on both the natural AR-dependent enhancer and promoter from the PSA gene and on a mini-promoter containing only two AREs. In addition, hZimp10 enhances endogenous AR-dependent activity in prostate cancer cells. These results provide the first line of evidence as to

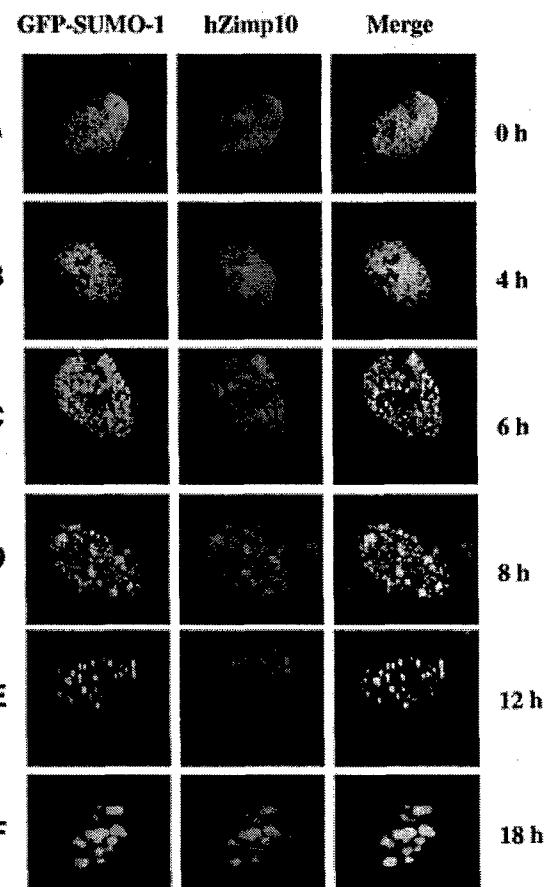


Fig. 9. Immunofluorescent co-localization of GFP-SUMO-1 and hZimp10 at DNA replication foci throughout S phase. CV-1 cells co-transfected with pcDNA3-Flag-hZimp10 and pGFP-SUMO-1 were synchronized with mimosine. Immunostaining was conducted with anti-Flag monoclonal antibody followed by secondary antibodies conjugated with rhodamine (red) to contrast with the GFP proteins. Merged images demonstrating co-localization of proteins are shown in the right panels (yellow). Staining was conducted at different time points as described in Figure 8.

the functional consequence of the interaction between AR and hZimp10.

Sequence analysis suggests that hZimp10 is related to the PIAS proteins. To determine the molecular basis by which hZimp10 functions as a transcriptional factor, we tested the intrinsic transcriptional activity of the protein. We showed that the C-terminal proline-rich domain possesses significant intrinsic transcriptional activity. Intriguingly, the latter activity is much higher than the TADs of other eukaryotic transcription factors that we have tested, including p53, Smad3, PU1 and AR, and is even comparable to transactivation by the TAD of VP16. These data, combined with the results from our transient transfection experiments, demonstrate that hZimp10 can enhance transcription both *in trans* and *in cis*, and are consistent with the notion that hZimp10 is a transcriptional regulator. Sequence analysis showed that the C-terminal proline-rich region is not found in other PIAS or PIAS-like proteins, suggesting that the Miz domain family may consist of a group of proteins that contain unique structures and play distinct roles in regulating transcription and other cellular processes.

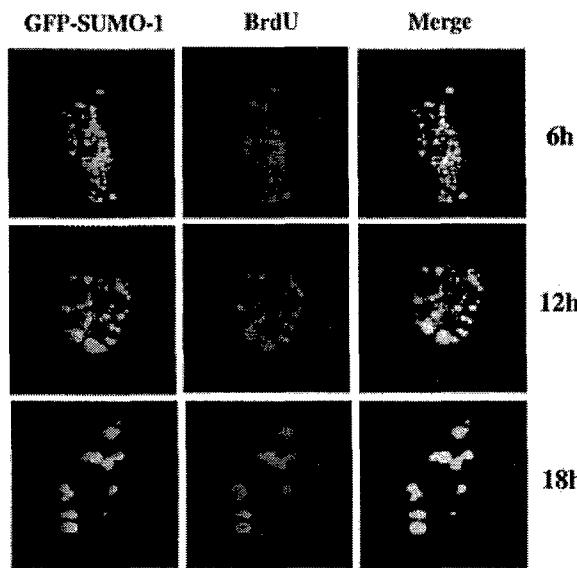


Fig. 10. Immunofluorescent localization of SUMO-1 at replication foci. CV-1 cells were transfected with GFP-SUMO-1 plasmid, and then synchronized as described in Materials and methods. Representative confocal laser scanning microscopic images of nuclei from cells expressing GFP-SUMO-1 proteins and pulsed with BrdU are shown. Color was developed by either FITC-conjugated monoclonal anti-BrdU antibody (red) or fluorescent images of GFP proteins (green). Merge (right panel) of left and middle panels indicates areas of co-localization (yellow).

Interestingly, the full-length hZimp10, when fused to the DBD of GAL4, displays a very limited activity compared with the truncated mutants containing the C-terminal proline-rich domain. We determined that the N terminus of hZimp10 (aa 1–691) significantly inhibits the activity of the C-terminal proline-rich region. The auto-inhibition of the transcriptional activity of hZimp10 indicates that a complicated regulatory mechanism may be involved in the enhancement of transcriptional activation by hZimp10. Therefore, identification of regulatory mechanisms that can release the inhibition and switch the protein from an inactive form into an active form will be extremely important.

Like other PIAS-like proteins, hZimp10 contains a conserved Miz domain. Another member of the PIAS family, ARIP3, was originally identified as an AR-interacting protein (Moilanen *et al.*, 1999). The zinc finger region of AR was shown to be involved in the interaction with ARIP3/PIASx α (Moilanen *et al.*, 1999). Other members of the PIAS protein family have also been shown to be capable of interacting with AR and other steroid receptors and to regulate their activity (Kotaja *et al.*, 2002; Tan *et al.*, 2002). Our data showing that the Miz region of hZimp10 is involved in the interaction with AR are consistent with previous reports, and suggest a biological role of hZimp10 in androgen signaling.

Using immunofluorescence imaging, we confirmed the interaction between hZimp10 and AR. In synchronized cells, both hZimp10 and AR were detected at replication foci and a significant amount of overlay between these two proteins was observed throughout S phase. In addition, we also demonstrated a co-localization of hZimp10 and

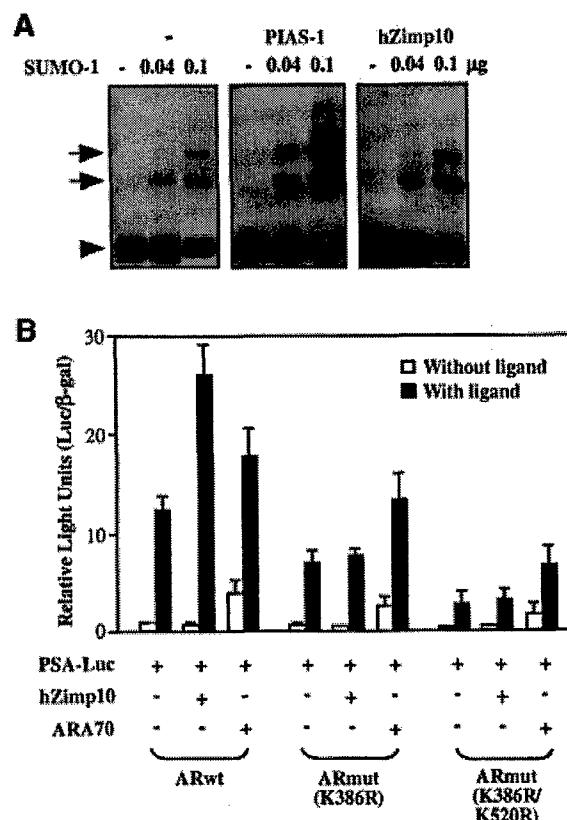


Fig. 11. hZimp10 enhances the sumoylation of AR and augments AR-mediated transcription. (A) COS-1 cells were co-transfected with 200 ng of pSG5-AR in the presence and absence of 200 ng of pFlag-PIAS1 or 360 ng of pcDNA3-Flag-hZimp10 along with 40 or 100 ng of pSG5-His-SUMO-1 as indicated. The cells were collected 48 h after transfection and the lysates were immunoblotted with the AR antibody. The slower migrating AR bands (sumoylated) are labeled with solid arrows. (B) CV-1 cells were transfected with a luciferase reporter driven by the 7 kb PSA promoter (100 ng), pcDNA3- β -gal (25 ng), pcDNA-hARwt or mut (5 ng) and pcDNA3-Flag-hZimp10 (10 ng). Cell lysates were prepared for assessment of luciferase and β -gal activities.

SUMO-1. These findings suggest a potential role for hZimp10 in both chromatin assembly and maintenance of chromatin. hZimp10 may act as a transcriptional regulator to initiate and mediate formation of an active transcriptional complex. It may also be involved in the modification of chromatin and participate in other steps during DNA synthesis. Interestingly, a novel *Drosophila* gene, termed the *tonalli* (*tta*), was identified recently (Gutierrez *et al.*, 2003), and shares some sequence similarity to hZimp10. Intriguingly, *tta* was shown to genetically interact with SWI2/SNF2 and the Mediator complex. These data are consistent with our discoveries, suggesting that hZimp10 may play a critical role in chromatin modification.

PIAS proteins can function as SUMO-1 E3 ligases to facilitate sumoylation of steroid receptors and other transcription factors (Kotaja *et al.*, 2002). It has been shown that the sumoylation of AR can be enhanced by PIAS proteins (Kotaja *et al.*, 2002) and two major sumoylation sites have been identified in the TAD of AR (Poukka *et al.*, 2000). Co-localization of hZimp10 with AR and SUMO-1 in the same subnuclear structures suggests that the sumoylation may play a role in the

interaction between the proteins. In this study, we further demonstrated that hZimp10 is able to enhance the sumoylation of AR. While the effect mediated by hZimp10 was relatively modest in comparison with PIAS1, it may reflect the fact that the role of hZimp10 in sumoylation is distinct from that of the PIAS proteins. Based on the experimental design, we were not able to conclude whether hZimp10 is acting directly as an E3 ligase. The mechanism by which hZimp10 enhances the sumoylation of AR needs to be investigated further. Transcription assays showed that in contrast to the wild-type AR, the activity of the sumoylation deficient AR mutant was not augmented by hZimp10. The above results not only provide evidence demonstrating that hZimp10 enhances the sumoylation of AR, but also demonstrate a link between the sumoylation and augmentation of AR activity by hZimp10. Although the precise mechanisms by which hZimp10-mediated sumoylation affects the transcriptional activity of AR remain unclear, our findings suggest several possibilities, one of which is that the sumoylation of AR may enhance the interaction between AR and hZimp10, and/or their localization to replication foci.

Recently, the PIAS and SUMO proteins have been found in a PML-related nuclear body (Schmidt and Muller, 2002). Our observation that hZimp10 and SUMO-1 co-localize to replication foci is novel and interesting. Given the recent result showing that PCNA is a target of SUMO-1 (Hoege *et al.*, 2002), sumoylation may be involved in both transcriptional regulation and DNA synthesis (Hoege *et al.*, 2002; Schmidt and Muller, 2002). In both cases, our discovery of hZimp10 and its link to the SUMO pathway provide a new line of evidence, suggesting that sumoylation, combined with other modification pathways such as methylation, phosphorylation and acetylation, is involved in regulating transcriptional activity of AR by modifying chromatin formation during the early and late S phases of the cell cycle.

Materials and methods

Yeast two-hybrid system

Yeast two-hybrid experiments were basically performed as described previously (Sharma *et al.*, 2000). The DNA fragment containing the partial TAD of human AR (aa 1–333) was fused in-frame to the GAL4 DBD in the pGBT9 vector (Clontech, Palo Alto, CA). The construct was transformed into a yeast strain PJ69-4A (James *et al.*, 1996). A cDNA library from the mouse EML cell line was used for this screening (Lioubin *et al.*, 1996). β -gal activities were measured using the Galacto-light Plus kit (Tropix Inc., Bedford, MA).

Plasmid construction

The C-terminal region of Zimp10 was isolated in the initial yeast two-hybrid screening. A BLAST search found KIAA1224 and several human EST cDNA clones that contained sequences similar to the mouse fragment. To isolate the full-length Zimp10, 5'-RACE was used with human brain and prostate Marathon Ready cDNAs (Clontech). The specific reverse primers spanning amino acid residues 464–472 (5'-CTGGCTTGTAACTACTGCCCATGTTG) and 176–184 (5'-TTCCCAAGGACCTGGCTCTGGATG) were used in first and second rounds of 5'-RACE, respectively. A full-length hZimp10 cDNA was created by combining the cDNA fragments coding for the N-terminal region isolated by 5'-RACE and the KIAA1224 cDNA fragment in the pcDNA3 vector. Subsequently, truncated mutants of hZimp10 were generated from the full-length clone.

The human AR plasmid, pSV-hAR, was provided by Dr Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands). A β -gal

reporter was purchased from Promega (Madison, WI). The human ER α plasmid and pERE-luc plasmid were supplied by Dr Myles Brown (Dana-Farber Cancer Institute, Boston, MA). A human PR construct (hPR1) and the PRE-luc reporter were provided by Dr Kathryn B. Horwitz (University of Colorado, Denver, CO). The expression constructs of human GR and VDR, and the pVDRE-luc reporter were gifts from Dr David Feldman (Stanford University, Stanford, CA). The pARE-luc reporter was a gift from Dr Chawnshang Chang (Yeh and Chang, 1996). The pPSA7kb-luc was obtained from Dr Jan Trapman (Cleutjens *et al.*, 1996). The SUMO-1 and ARIP3 vectors were generated as reported previously (Poukka *et al.*, 2000; Kotaja *et al.*, 2002). Luciferase reporters containing the chicken myelomonocytic growth factor gene minimal promoter were provided by Dr Donald Ayer (University of Utah, Salt Lake City, UT).

Cell culture and transfections

The monkey kidney cell line, CV-1, and other human cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (HyClone, Denver, CO). An AR-positive prostate cancer cell line, LNCaP, was maintained in T-medium (Invitrogen, Carlsbad, CA) with 5% fetal calf serum. Transient transfections were carried out using LipofectAMINE for CV-1 cells, and LipofectAMINE 2000 for LNCaP cells (Invitrogen). The RLU from individual transfections were normalized using β -gal activity in the same samples. Individual transfection experiments were done in triplicate and the results are reported as mean RLU/ β -gal (\pm SD).

Immunoprecipitation, western blotting and antibody production

pSV-hAR alone or with the Flag-tagged hZimp10 and GFP-SUMO-1, was transfected into CV-1 cells. Co-immunoprecipitation assays and western blotting were carried out as described previously (Sharma *et al.*, 2000). The membranes were then probed with a polyclonal antibody against the N-terminus of AR (Santa Cruz Biotech, Santa Cruz, CA), or a monoclonal Flag tag antibody (Sigma, St Louis, MO). Proteins were detected using the ECL kit (Amersham, Arlington Heights, IL).

The C-terminus of hZimp10 (aa 917–1067) was inserted into the pGEX-4T1 vector (Amersham). The GST fusion protein was prepared as described previously (Smith and Johnson, 1988). The GST-hZimp10 fusion protein was used as a source of antigen for antibody production. The antibody was produced and purified by BioChain Institute Inc. (Hayward, CA).

Northern blot analysis

A blot with RNA from multiple human tissues was obtained from Clontech Inc. Poly(A)⁺ RNAs were isolated by Oligo-dT (Invitrogen) chromatography from prostate cancer cell lines (LNCaP, DU145, PC-3, ARCaP and LAPC4) and non-prostate cancer cell lines (MCF7 and HeLa). Northern blots were performed as described previously (Sharma *et al.*, 2000).

Cell synchronization, BrdU labeling and immunofluorescence

CV-1 cells were seeded onto gelatin-coated (0.2%) coverslips 24 h before transfection. Transfected cells were fed with the fresh medium after 6 h and then incubated for an additional 12 h before synchronization (Krude, 1999). For detection of DNA replication, cells were pulsed with 10 μ M 5-BrdU and 1 μ M fluorodeoxyuridine (Sigma) to inhibit thymidylate synthetase. Cells were then washed twice with cold PBS and fixed with 3% formaldehyde for 30 min. After fixation, cells were washed again. To visualize the newly synthesized DNA labeled with BrdU, the rinsed permeabilized cells were treated with 4 N hydrochloric acid to denature the DNA, rinsed several times in TBS-T, and incubated at 37°C for 1 h with FITC-conjugated monoclonal anti-BrdU antibody (PharMingen, San Diego, CA).

At various time points after synchronization, cells grown on coverslips were fixed with 3% formaldehyde for 30 min at 22°C, permeabilized with 0.1% Nonidet P-40 in PBS, and blocked with 5% milk in 1× TBS-T for 30 min at room temperature. The cells were then incubated with monoclonal anti-Flag or polyclonal anti-AR antibodies alone or together at a 1:1000 dilution in 1% BSA/PBS for 1 h at 22°C. Cells were washed three times followed by incubation with fluorescein isothiocyanate-conjugated or rhodamine-conjugated anti-mouse, or rhodamine-conjugated anti-rabbit secondary antibody, respectively (Molecular Probes, Eugene, OR).

Immunohistochemistry

Human prostate tissue samples were fixed in 10% neutral buffered formalin, and processed to paraffin. Sections were cut at 5 μ m intervals, de-waxed in Histoclear (National Diagnostic, Atlanta, GA), and then hydrated in graded alcoholic solutions and distilled water. Two adjacent sections from each tissue sample were used for immunohistochemistry. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol (ImmunoVision Technology, Springdale, AR). The sections were then incubated with either the rabbit polyclonal anti-AR antibody (Affinity BioReagents, Golden, CO) at a 1:100 dilution, or the chicken anti-hZimp10 antibody at a 1:150 dilution overnight at 4°C. Sections were washed and incubated with a 1:200 dilution of either a biotinylated anti-rabbit (Zymed Corp., South San Francisco, CA) or anti-chicken antibody (Amersham), and then washed and developed with DAB in PBS and 0.03% H₂O₂. All sections used for immunohistochemistry were lightly counterstained with 5% (w/v) Harris hematoxylin.

SUMO-1 conjugation

The experiment was performed essentially as described previously (Kotaja *et al.*, 2002). COS-1 cells seeded onto 6-well plates were transfected with 200 ng of pSG5-AR in the presence and absence of 200 ng of pFlag-PIAS1 or 360 ng of pcDNA3-Flag-hZimp10 along with 40 ng or 100 ng of pSG5-His-SUMO-1 as indicated. The cells were supplied with 100 nM testosterone 24 h after transfection, collected 48 h after transfection and lysed in RIPA-1 buffer containing 10 mM *N*-ethylmaleimide. The lysates were immunoblotted and developed with the anti-AR antibody.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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Mechanism of p21-activated Kinase 6-mediated Inhibition of Androgen Receptor Signaling*

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PAK6 was first identified as an androgen receptor (AR)-interacting protein able to inhibit AR-mediated transcriptional responses. PAK6 is a serine/threonine kinase belonging to the p21-activated kinase (PAK) family implicated in actin reorganization and cell motility, gene transcription, apoptosis, and cell transformation. We investigated the biochemical basis for inhibition of AR signaling by PAK6. We compared the kinase activity of PAK6 with two other well characterized members of the PAK family, PAK1 and PAK4. Like PAK4, PAK6 possesses a constitutive basal kinase activity that, unlike PAK1, is not modulated by the binding of active Rac or Cdc42 GTPases. In order to test the involvement of PAK6 kinase activity in suppression of AR-mediated transcription, we generated kinase-dead (K436A) and kinase-active (S531N) mutants of PAK6. We show that PAK6 kinase activity is required for effective PAK6-induced repression of AR signaling. Suppression does not depend upon GTPase binding to PAK6 and is not mimicked by the closely related PAK1 and PAK4 isoforms. Kinase-dependent inhibition by PAK6 extended to the enhanced AR-mediated transcription seen in the presence of coactivating molecules and to the action of AR coinhibitors. Active PAK6 inhibited nuclear translocation of the stimulated AR, suggesting a possible mechanism for inhibition of AR responsiveness. Finally, we observe that autophosphorylated, active PAK6 protein is differently expressed among prostate cancer cell lines. Modulation of PAK6 activity may be responsible for regulation of AR signaling in various forms of prostate cancer.

Currently, six members of the p21-activated kinase (PAK)¹ family of protein kinases have been identified and can be clas-

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¹ The abbreviations used are: PAK, p21-activated kinase; AR, androgen receptor; ARA, androgen receptor-associated; β -gal, β -galactosidase; CRIB, Cdc42/Rac-interactive binding; DBD, DNA binding domain; DHT, dihydrotestosterone; LBD, ligand binding domain; Luc, luciferase; GST, glutathione S-transferase; TAD, transcription activation domain; GTP γ S, guanosine 5'-3-O-(thio)triphosphate; wt, wild type; DTT,

sified into two groups based on their sequence homology and regulatory properties: group I, including PAKs 1–3, and group II, including PAKs 4–6 (1–3). PAKs are serine/threonine kinases that contain a Cdc42/Rac-interactive binding (CRIB) domain and a Ste20-related kinase domain. The PAK family members have been implicated in the regulation of multiple cellular functions, including actin reorganization, cell motility, gene transcription, cell transformation, apoptotic signaling, and more recently, steroid hormone receptor signaling (see below).

The binding of activated GTP-bound Cdc42 or Rac to group I PAKs dramatically stimulates their ability to phosphorylate exogenous substrates. In contrast, the group II PAKs, PAK4 and PAK5, possess a substantial "basal" kinase activity that is not further stimulated by binding of activated GTPase (4, 5). GTPase binding does mediate kinase relocation: after binding Cdc42, PAK4 is relocalized to the Golgi (4), and PAK5 shuttles from the microtubule network to actin-rich structures (6). The mechanisms by which PAK6 activity is regulated and the role of PAK6 kinase activity in its biological functions have not yet been studied.

PAK6 was identified by yeast two-hybrid screening as an androgen receptor-interacting protein (7). After androgen stimulation, PAK6 was reported to interact with the ligand binding domain of the androgen receptor (AR) and to translocate to the nucleus along with the AR, where PAK6 inhibits AR-mediated transcription. Northern blot analysis shows that PAK6 is mainly expressed in brain, testis, prostate, and breast tissues (7, 8). PAK6 has also been shown to bind the estrogen receptor (ER) and to inhibit ER-mediated gene transcription (8). Interestingly, the inhibitory effect of PAK6 on AR- and ER-mediated gene transcription is opposite to the transactivation of the ER induced by PAK1-mediated receptor phosphorylation (9).

The AR and ER are hormone-activated transcription factors that belong to the nuclear receptor superfamily (10, 11). The AR has a fundamental role in the development and differentiation of androgen-sensitive tissues and also plays an important role in the pathogenesis of prostate cancer (12). Structurally, the AR is composed of three important functional domains, an N-terminal transactivation domain (TAD), a DNA binding domain (DBD), and a ligand binding domain (LBD). In the absence of androgen, the AR is localized in the cytoplasm in association with heat shock proteins (13, 14). Upon stimulation by dihydrotestosterone (DHT) or DHT analogues, including R1881, heat shock proteins are released, and homodimerization and translocation of the AR to the nucleus occur (15, 16).

dithiothreitol; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; ER, estrogen receptor; MMTV-LTR, murine mammary tumor virus-long term repeat.

The ligand-bound nuclear AR is capable of mediating transactivation and/or transcriptional repression of target genes. The transcriptional activity of AR is modulated by interaction with cofactors, including coactivators that enhance AR activity and corepressors that inhibit AR activity (17). SRC1, p300, Tip60 α , β -catenin, and ARA55 are among these coactivating molecules that have been shown to increase AR-mediated transcription (18–23). The transcriptional activity of the AR can also be regulated either by direct phosphorylation of the receptor and/or by phosphorylation of cofactors (24–28).

In this paper, we investigated the mechanism(s) by which PAK6 inhibits AR-mediated transactivation. By using both kinase-inactive and constitutively kinase-active mutants of PAK6, we showed that inhibition of AR transcriptional activity by PAK6 is dependent on its kinase activity. The binding of Cdc42 GTPase to PAK6 was not required for transcriptional inhibition. The inhibitory effect of PAK6 on AR-mediated transcription was dominant to AR coactivator functions and synergized with corepressors. PAK6 phosphorylated the DNA binding domain of the AR in *in vitro* kinase assays. Finally, we observed that phosphorylated, activated PAK6 protein is expressed differently among the different prostate cancer cell lines. Our results suggest that modulation of PAK6 expression and/or kinase activity may be an important component in the regulation of AR signaling in various forms of prostate cancer.

EXPERIMENTAL PROCEDURES

Materials—Cell culture media, fetal bovine serum, and supplements were from Invitrogen. [γ -³²P]ATP (specific activity 4500 mCi/mmol) was from ICN, Costa Mesa, CA. Plasmids for transfection were purified using the Qiafilter purification system of Qiagen, Chatsworth, CA. Thrombin was purchased from Sigma; GDP and GTP γ S were from PerkinElmer Life Sciences. For PCR, the Expand High Fidelity PCR system from Roche Applied Science was used.

Cell Cultures—Human cancer cell lines PC-3, PC3MM2 (41), Du145, and HeLa were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (25 units/ml), and streptomycin (25 μ g/ml). MCF-7, ARCaP (42), and LNCaP were maintained in RPMI 1640 with 10% fetal calf serum, penicillin (25 units/ml), and streptomycin (25 μ g/ml). PC3zj is a subclone of the human prostate carcinoma cell line PC3.

Plasmid Construction—PAK1, PAK4, and PAK6 were subcloned into pcDNA3-EGFP. PAK6 was inserted into the Myc tag vector pCMV6M. To produce K436A, S531N, and S560E single mutations in PAK6, overlapping PCR was performed using outer boundary primers (PAK6-EcoRI-5' and PAK6-XhoI-3') and overlapping primer pairs to introduce the desired mutations (for K436A, forward primer 5'-CGC CAG GTG GCC GTC gca ATG ATG GAC CTC AGG-3'; reverse primer 5'-CCT GAG GTC CAT CAT tgc GAC GGC CAC CTG GCG-3'; for S531E, forward primer 5'-GAC ATC AAG AGT GAC aac ATC CTG CTG ACC-3'; reverse primer 5'-GGT CAG CAG GAT gtt GTC ACT CTT GAT GTC-3'; for S560N, forward primer 5'-GTC CCT AAG AGG AAG gag CTG GTG GGA ACC CCC-3'; reverse primer 5'-GGG GGT TCC CAC CAG ctc CCT CTT AGG GAC-3'; and wt PAK6 as a template (lowercase letters indicate the introduced base mutation)). The different fragments were inserted into EcoRI/XhoI-cut pcDNA3-EGFP. To produce the S531N,S560E double mutations in PAK6, overlapping PCR was performed using outer boundary primers (PAK6-EcoRI-5' and PAK6-XhoI-3'), the overlapping primer pairs S560N, and using PAK6 S531N as a template. The fragment was inserted into EcoRI/XhoI-cut pcDNA3-EGFP. The mutations H20L,H23L were introduced by site-directed point mutation using Quickchange kit (Stratagene, La Jolla, CA) according to the manufacturers instruction. Primer sequences are as follows: PAK6(H20L,H23L) 5'-CCA CAG AAC TTC CAG CTC CGT CTC CTC ACC TCC TTC-3' and its complementary sequence. The reporter plasmid MMTVpA3-Luc, containing the luciferase gene under the control of the steroid hormone-response elements in the MMTV-LTR, was provided by Dr. Richard Pestell (Albert Einstein Medical College, New York). The human AR cDNA, cloned into SV40 promoter-driven expression vector, pSV-hAR, was provided by Dr. Albert Brinkmann (Erasmus University, Rotterdam, The Netherlands). An SV40-driven β -galactosidase reporter plasmid (pSV- β -GAL) (Promega, Madison, WI) was used in this study as an internal control. The mouse

SMRTa cDNA, cloned into pCMX, was provided by Dr. Ronald M. Evans (The Howard Hughes Medical Institute, and the Salk Institute for Biological Studies, La Jolla, CA).

Purification of Recombinant Proteins—GST-AR (TAD, DBD, and LBD) and Cdc42 proteins in vector pGEX-2TK were expressed in BL21 cells and purified according to the protocol of Amersham Biosciences. Buffers for the GST-Cdc42 purification contained 1 μ M GDP starting from the lysis step, excluding the dialysis buffer. The GST moiety was cleaved off Cdc42 with thrombin at a final concentration of 10 units/ml glutathione beads. Thrombin was removed by incubation with *p*-aminobenzamidine beads (Sigma), and the protein was dialyzed four times against 25 mM Tris/HCl, pH 7.5, 1 mM EDTA, 5 mM MgCl₂, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride. After dialysis, 1 μ M GDP was added again, and the purified protein was concentrated by ultrafiltration.

Immunoprecipitation—HeLa cells were seeded on 10-cm cell culture dishes at 50–70% confluence and transfected using LipofectAMINE (Invitrogen). 2 μ g of plasmid DNA and 30 μ l of LipofectAMINE were used per dish, and the transfection protocol was essentially followed according to the manufacturer's guidelines (Invitrogen). After 30 h the cells were washed with PBS, lysed in 0.5 ml of Lysis Buffer (25 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 1 mM DTT, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride), and centrifuged for 5 min at 4 °C at 15,000 \times g, and the supernatant was collected. Protein expression in the lysates was determined by immunoblotting. For precipitations of EGFP, Myc, or FLAG-tagged PAK6 proteins, lysate containing equal amounts of the proteins was incubated with equilibrated protein G beads and anti-EGFP (3E6; Molecular Probes, Eugene, OR), anti-Myc (9E10; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-FLAG (M2; Molecular Probes) antibodies for at least 3 h or overnight at 4 °C. The bead fraction was washed four times with lysis buffer, twice in kinase buffer, and used for kinase assays.

Loading of Cdc42—Cdc42 was loaded with GTP γ S or GDP under the following conditions. 5–20 μ g of Cdc42 was incubated in 25 mM Hepes/NaOH, pH 7.5, containing 20 mM EDTA and 1 mM GTP γ S or GDP for 10 min at 30 °C in a total volume of 25–100 μ l. The reaction was stopped by addition of MgCl₂ at 100 mM final concentration. GTP γ S or GDP-loaded Cdc42 was directly used in kinase or pull-down assay.

In Vitro Kinase Assay—Kinase reactions with immunoprecipitated PAK were performed in kinase buffer (50 mM Hepes/NaOH, pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂, 0.2 mM DTT) in a volume of 60 μ l with 250 μ M ATP. Radiolabeled ATP was used at 10 μ Ci/reaction. The reactions were incubated for 30 min at 30 °C and stopped by addition of sample buffer. Histones H3/H4 or AR proteins were used as a substrate at 1 μ g/reaction.

Western Blot—Cells were lysed and supernatants collected as described above. β -Mercaptoethanol and bromphenol blue were added, and cell lysate proteins were resolved by SDS-PAGE. Proteins were then electroblotted onto nitrocellulose filters, and filters were blocked by incubation for 1 h with 5% bovine serum albumin in Tris-buffered saline, 0.1% Tween 20 and then incubated overnight at 4 °C with anti-EGFP antibody (rabbit antibody, Molecular Probes, Eugene, OR) or anti-PAK6 phospho-antibody (anti-PAK4(Ser-474) anti-PAK5(Ser-602), and anti-PAK6(Ser-560) phospho-antibody 3241; Cell Signaling Technology, Inc. Beverly, MA). Blots were washed three times for 10 min in Tris-buffered saline plus 0.1% Tween 20 and incubated for 1 h with peroxidase-labeled anti-rabbit immunoglobulins. Blots were developed with the use of the enhanced chemiluminescence detection system (Pierce).

Pull-down Assay—Cells were transfected and lysed, and supernatants were collected as described above. Clarified lysate was incubated with either 1 μ g of GTP γ S or GDP-loaded Cdc42 for 2 h at 4 °C. Anti-EGFP antibody and protein G beads were then added and incubated at 4 °C for another hour. Beads were washed three times in lysis buffer, boiled in SDS sample buffer for 5 min, separated by SDS-PAGE, and subjected to a Western blotting with either an EGFP antibody (rabbit antibody, Molecular Probes) or a Cdc42 antibody (SC-87, Santa Cruz Biotechnology).

AR Transcriptional Activity—The monkey kidney cell line CV-1 containing no detectable levels of endogenous steroid hormone receptor activity was maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (Hyclone, Denver, CO). Transient transfections were carried out with LipofectAMINE (Invitrogen). Cells were plated on 12-well plates at 50–70% confluence 16 h before transfection, and 400 ng of total plasmid DNA per well was used in the transfection. Approximately 16 h after transfection, the cells were washed and fed with medium containing 5% charcoal-stripped (steroid

hormone-free) fetal calf serum (Hyclone) in the presence or absence of R1881. Cells were incubated for another 24 h, lysates prepared, and luciferase and galactosidase activities measured with the Luciferase Assay kit (Promega) and Galactolight kit (Tropix, Bedford, MA), respectively.

Immunofluorescence—HeLa cells were plated onto 2-well chamber slides. After 24 h, cells were transiently transfected with wt or mutated EGFP-tagged pcDNA3-PAK6 and pSV-hAR with LipofectAMINE PLUS reagent (Invitrogen) according to the manufacturer's instructions. 24 h post-transfection, cells were left untreated or treated with 10 nM R1881 for another 24 h. Cells were fixed for 5 min with 4% paraformaldehyde in PBS and placed in methanol for 1 min, followed by sequential washes with PBS and 1% fetal bovine serum in PBS. Cells were incubated with polyclonal anti-AR antibody (Santa Cruz N20816) for 1 h at room temperature. Cells were washed with PBS and incubated with Texas Red-conjugated anti-rabbit secondary antibody (Molecular Probes). Cells were dried and mounted with fluor mounting medium (Vector Laboratories). Pictures were taken using confocal microscopy.

RESULTS

Analysis of PAK6 Kinase Activity—In order to evaluate the kinase activity of PAK6 and compare it to different members of the PAK family, HeLa cells were transfected with vectors containing wild type (wt) PAK6, PAK1, or PAK4. Equal amounts of EGFP-PAK1wt, EGFP-PAK6wt, and EGFP-PAK4wt were immunoprecipitated with an anti-EGFP antibody, and an *in vitro* kinase assay was performed using histone H3/H4 as an exogenous substrate, in the presence of either GDP-loaded (inactive) or GTP γ S-loaded (active) Cdc42. As expected, significant phosphorylation of H3/H4 by PAK1wt was stimulated only in the presence of Cdc42-GTP γ S. In contrast, substantial phosphorylation of H3/H4 by PAK6wt and PAK4wt was observed in the presence of both Cdc42-GDP and Cdc42-GTP γ S, and the activity was not enhanced by the presence of the active GTPase (Fig. 1A). The relative kinase activity of Cdc42-activated PAK1wt was stronger than with PAK6wt or PAK4wt at comparable protein expression levels. An additional slower migrating band was observed with PAK6wt, suggesting that, as with PAK1 and PAK4wt, PAK6wt became autophosphorylated. However, unlike the autophosphorylation of PAK1wt, which is activation (Cdc42-GTP γ S)-dependent, autophosphorylation of PAK6wt and PAK4wt was Cdc42-independent.

In order to verify that the presence of the relatively large EGFP tag did not alter the ability of Cdc42 to activate PAK6, HeLa cells were transfected with vectors containing Myc-, FLAG-, or EGFP-tagged versions of PAK6wt, and then protein was immunoprecipitated with the relevant epitope tag antibody. An *in vitro* kinase assay was performed in the presence of Cdc42 loaded with either GDP or GTP γ S using histone H3/H4 as an exogenous substrate. As shown in Fig. 1B, phosphorylation of H3/H4 mediated by each FLAG-, Myc-, and EGFP-tagged PAK6wt was not modulated by the presence of Cdc42 loaded with either GDP or GTP γ S. These results show that PAK6 possesses a substantial basal kinase activity that is not further stimulated by Cdc42 (or Rac; data not shown).

Characterization of PAK6 Mutants—Several PAK6 mutants were generated in an attempt to obtain kinase-inactive and highly kinase-active forms of PAK6. PAK6 (K436A) introduced a lysine-to-alanine mutation at amino acid 436 in the activation loop within the kinase domain (Fig. 2A). Mutation of this residue is predicted to inhibit the binding of ATP and abolish kinase activity. PAK6(S560E) converted serine in a predicted autophosphorylation site (based on homology to PAK1) to glutamic acid. PAK6(S531N) introduced a serine-to-asparagine mutation at amino acid 531 in the catalytic loop. Mutation of this residue is predicted to stabilize the catalytic loop, as is the double mutant PAK6(S531N,S560E) (Fig. 2A). The EGFP-tagged version of each construct was transiently transfected into HeLa cells; equal amounts of PAK6 protein were immuno-

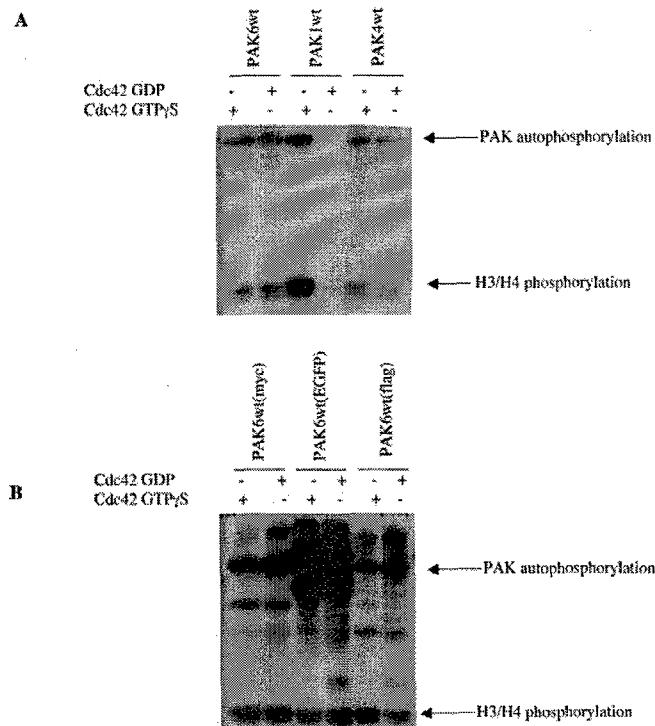


FIG. 1. Analysis of PAK6 kinase activity. *A*, HeLa cells were transiently transfected with pcDNA3-EGFP expression vector containing wild type PAK1, PAK4, or PAK6. After cell lysis, the amount of each PAK in the lysate was evaluated by Western blot and densitometry of the common EGFP fusion. Equal amounts of each PAK were incubated with Cdc42 loaded with either GDP or GTP γ S, and an *in vitro* kinase assay was performed using histone H3/H4 as substrate. Phosphorylation was detected by autoradiography. *B*, HeLa cells were transfected with pcMV-myc, pcDNA3-EGFP, or pcDNA3-FLAG expression vectors containing PAK6wt. PAK6wt Myc-tagged, EGFP-tagged, or FLAG-tagged proteins were immunoprecipitated with an anti-Myc, anti-EGFP, or anti-FLAG antibody, respectively. Immunoprecipitates were incubated with Cdc42 loaded with GDP or GTP γ S, and kinase assays were performed using histone H3/H4 as substrate. Phosphorylations were detected by autoradiography. Results shown are indicative of 3–4 similar experiments.

purified from cell lysates using EGFP antibody, and an *in vitro* kinase assay was performed using histone H3/H4 as an exogenous substrate. As shown in Fig. 2B, PAK6(K436A) exhibited no autophosphorylation nor exogenous kinase activity. Mutation S560E alone did not change the exogenous kinase or autophosphorylation activity of PAK6 from that of PAK6wt. In contrast, the mutation S531N strongly enhanced both exogenous kinase and autophosphorylation activity of PAK6. A similarly enhanced PAK6 activity was observed with the PAK6(S531N,S560E) double mutant. These results suggest that the S531N mutation stabilized the catalytic loop within the kinase domain of PAK6 to increase the kinase activity. The kinase activity of these mutants was not modulated by the presence of Cdc42 loaded with either GDP or GTP γ S (Fig. 2C).

The ability of an anti-phospho-PAK6 antibody directed against the Ser-560 predicted autophosphorylation site to detect active PAK6 was evaluated. Phosphorylation of the corresponding serine 423 residue in PAK1 was observed after activation by Rac/Cdc42 and reflected PAK1 kinase activity (29–31). HeLa cells were transfected with PAK6wt, K436A, S531N, and S560E, and cell lysates were prepared and analyzed by Western blot using anti-phospho-PAK6 antibody (Fig. 2D, *upper panel*). An anti-EGFP antibody was used to confirm similar protein expression levels (Fig. 2D, *lower panel*). As expected, PAK6(K436A) kinase-inactive and PAK6(S560E)-modified serine were not detected by the anti-phospho-PAK6 antibody. In

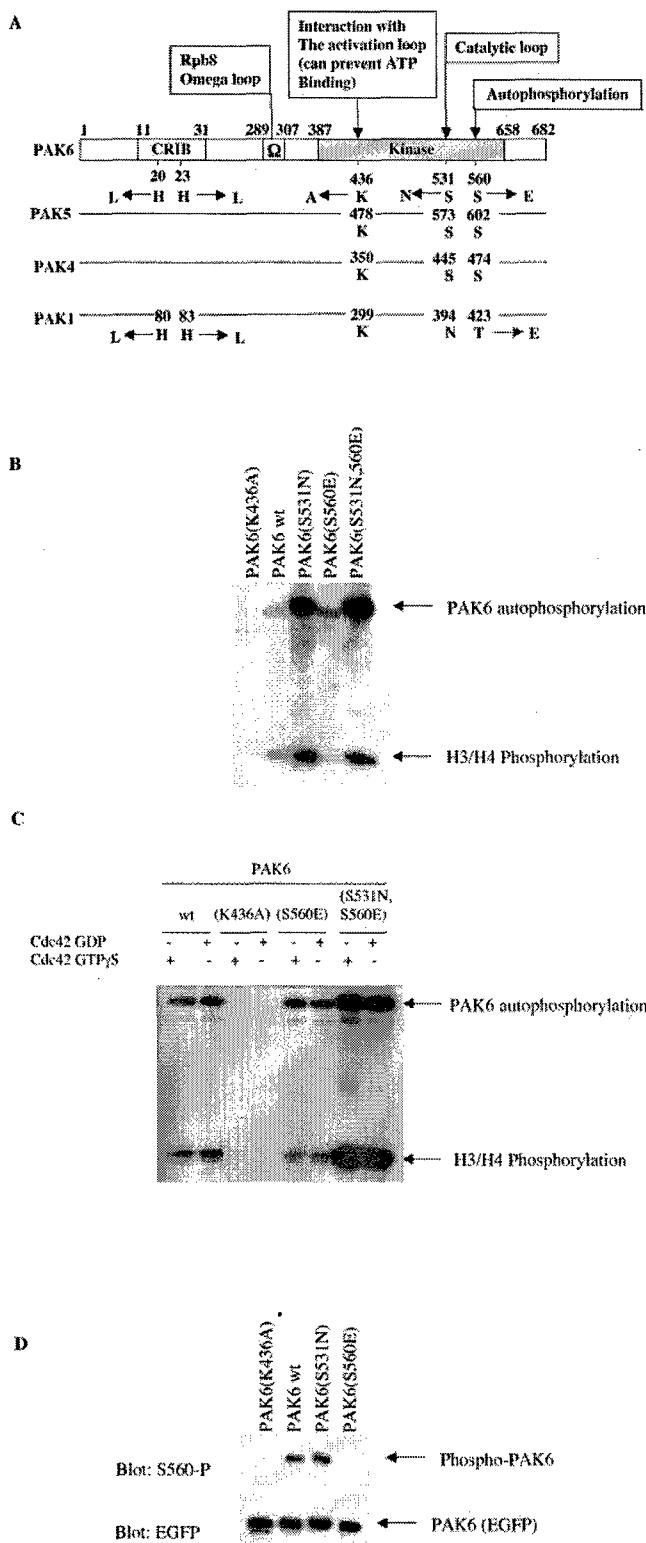


FIG. 2. Kinase activity of PAK6 mutants. *A*, schematic representation of PAK6 showing functional domains and point mutations used in this study. An alignment of the kinase domain and the CRIB domain of PAK1, PAK4, PAK5, and PAK6 shows the position of conserved amino acids. Mutation PAK6(K436A) corresponds to the kinase-inactivating mutation of the ATP-binding site K299A in PAK1. Mutation PAK6(S531N) corresponds to the activating mutation in the catalytic loop S445N in PAK4. The mutation S560E in PAK6 corresponds to the activating mutation of the autophosphorylation site T423E in PAK1. The mutations H20L,H23L in PAK6 correspond to mutations H83L,H86L in the CRIB domain of PAK1, which has been shown to abolish the binding of Rac or Cdc42 to PAK1 (3, 33). The Rpb8 Ω loop

contrast, this antibody was able to detect PAK6wt and PAK6(S531N) mutants. These results are consistent with phosphorylation at serine 560 as an indicator reflecting PAK6 kinase activity.

Repression of AR-mediated Transcription by PAK6 Is Dependent on PAK6 Kinase Activity—It has been shown previously (7, 8) that overexpression of PAK6 specifically repressed AR-mediated transcription. To assess whether the kinase activity of PAK6 was required to inhibit AR-mediated transcription, CV1 cells were cotransfected with AR (pSVAR) and PAK6 plasmids, along with a luciferase gene reporter under the control of the steroid hormone-responsive elements in the MMTV-LTR (MMTVpA3-Luc). As shown in Fig. 3A, PAK6wt was able to repress in a dose-dependent manner AR transcriptional activity induced by different concentrations of R1881 ranging from 0.1 to 100 nM. wt PAK6 and mutant (S560E), which both have the same relative level of kinase activity, repressed to a similar extent AR transcriptional activity induced by R1881 (Fig. 3B). Significantly, PAK6(S531N) and PAK6(S531N,S560E) mutants that exhibited strong kinase activities inhibited ligand-dependent AR transcriptional activity more effectively (*i.e.* by more than 85%) at similar levels of transfected DNA. In marked contrast, kinase-inactive mutant PAK6(K436A) inhibited less than 10–20% of AR-dependent transcriptional activity (Fig. 3B). There is thus a correlation between relative kinase activity of PAK6 proteins and their ability to suppress AR-mediated transcription.

We next tested the specificity of the inhibition of AR transcriptional activity by transfecting CV1 cells with wt or dominant active versions of PAK1, PAK4, and PAK6. Fig. 3C shows that PAK4 only weakly repressed AR transcriptional activity, whereas PAK1 modestly stimulated AR transcriptional activity. PAK6(S531N) dominant active was used as control. These results provide evidence that the inhibition of AR-mediated transcription is specific to the testis/prostate-enriched PAK6 isoform.

PAK6 Inhibition of AR-mediated Signaling Is Independent of GTPase Binding—The Rho family GTPase Cdc42 has been shown to bind to PAK6, presumably via its CRIB domain (9, 32), but we have demonstrated that this interaction does not affect PAK6 kinase activity. The binding of Cdc42 to PAK4 and PAK5 also does not increase their kinase activity but rather modulates their subcellular localization (4, 6). We wanted to determine whether the interaction of PAK6 with GTPase was important in enabling PAK6 to inhibit AR-mediated transcription. We generated a series of PAK6 constructs in which the CRIB domain was mutated in a conserved pair of histidine residues known to be important for GTPase binding (PAK6(H20L,H23L), PAK6(H20L,H23L,K436A), and PAK6(H20L,H23L,S531N)) (33). The binding of Cdc42 to these PAK6 constructs was analyzed by immunoprecipitation of EGFP-tagged PAK6 mutants in the presence of Cdc42 loaded

domain of PAK6, homologous to RNA polymerase subunit 8 Omega loop, is also shown. *B*, HeLa cells were transfected with pcDNA3-EGFP expression vector containing PAK6wt or PAK6 mutants K436A, S531N, S560E, and S531N,S560E. An *in vitro* kinase assay using histone H3/H4 as substrate was performed on the immunoprecipitated PAK6 proteins and then analyzed by autoradiography. *C*, HeLa cells were transfected with pcDNA3-EGFP expression vector containing PAK6wt or PAK6 mutants as indicated. PAK6 proteins were immunoprecipitated and incubated with Cdc42 loaded with GDP or GTP γ S, and a kinase assay was performed. Phosphorylations were detected by autoradiography. *D*, HeLa cells were transfected with pcDNA3-EGFP expression vector containing PAK6wt or PAK6 mutants K436A and S531N,S560E. An aliquot of each lysate was analyzed by Western blot using an anti-EGFP antibody (*top panel*) or an antibody recognizing PAK6 protein phosphorylated on serine 560 (*bottom panel*). Results shown in *B–D* are representative of three similar experiments.

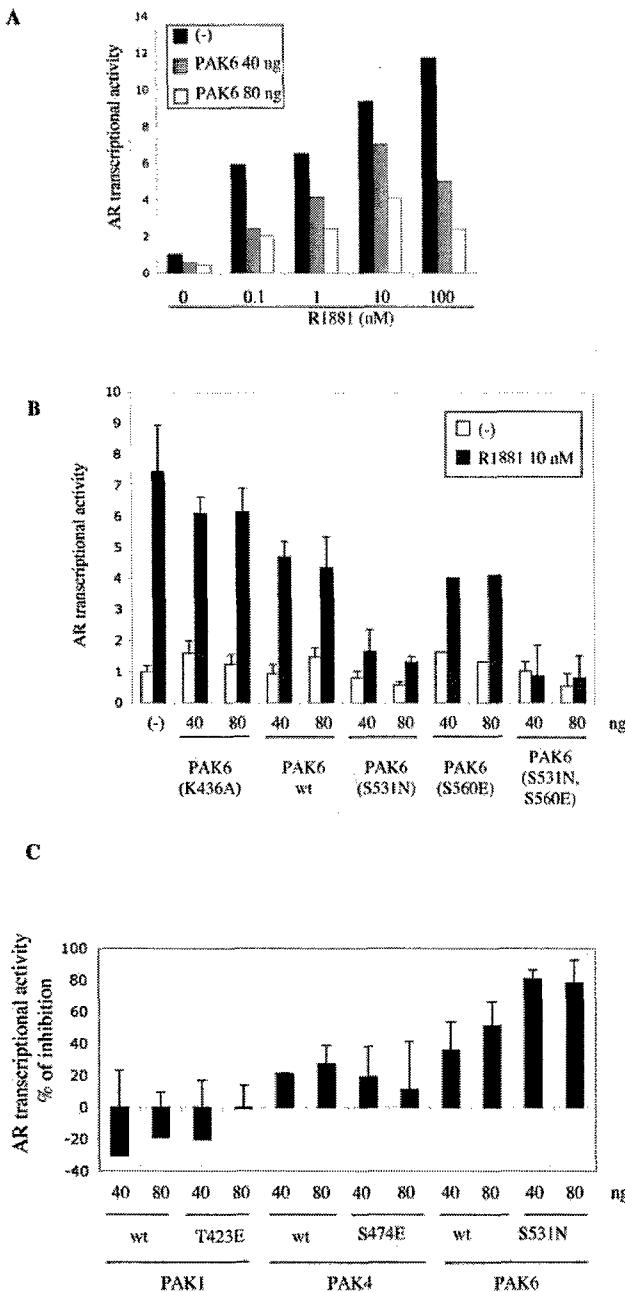


FIG. 3. Repression of AR-mediated transcription by PAK6 is dependent on PAK6 kinase activity and is specific to PAK6. *A*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40- β -gal, 40 ng of pSV-hAR, and 40 or 80 ng of pcDNA3-EGFP-PAK6 constructs. The total amount of DNA was normalized with pcDNA3. 18 h after transfection, cells were treated with the indicated concentrations of R1881 (0.1–100 nM) and cell extracts prepared 24 h after stimulation, and luciferase activity was monitored. Luciferase activity is reported as relative light units normalized to β -galactosidase activity. *B*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40- β -gal, 40 ng of pSV-hAR, and 40 or 80 ng of pcDNA3-EGFP expression vector containing PAK6 mutants K436A, wt, S531N, S560E, and S531N,S560E cDNAs. 18 h after transfection cells were treated with 10 nM R1881 (gray bars) or left untreated (white bars), and luciferase activity was determined (reported as relative light units). *C*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40- β -gal, 40 ng of pSV-hAR, and 40 or 80 ng of expression vector containing wild type (wt) PAK1, -4, -6 or active kinase mutant PAK1(T423E), PAK4(S474E), and PAK6(S531N). Total amount of DNA was normalized with pcDNA3. 18 h after transfection, the cells were stimulated with 10 nM R1881 for another 24 h and cell extracts prepared, and luciferase activity was determined. The bar graphs shown in *B* and *C* represent the mean \pm S.E. % inhibition of AR-mediated transcription observed in three separate experiments.

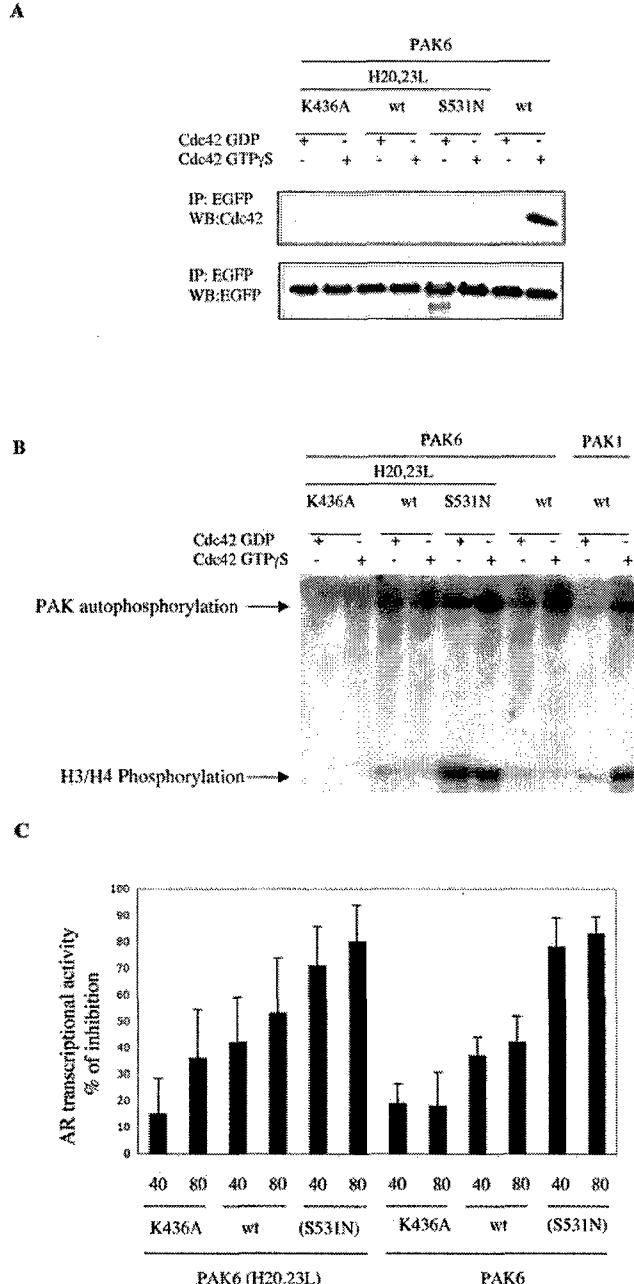


FIG. 4. PAK6 inhibition of AR-mediated transcription is independent of Cdc42 binding. *A*, binding of GTPyS loaded Cdc42 to PAK6. HeLa cells were transfected with pcDNA3-EGFP expression vector containing either PAK6wt or PAK6 mutants H20L, H23L, K436A, H20L, H23L, and H20L, H23L, S531N cDNAs. Equal amounts of cell lysate were incubated with Cdc42 loaded with either GDP or GTPyS and immunoprecipitated with an anti-EGFP antibody. The precipitated fractions were then resolved by SDS-PAGE and analyzed by Western blot using an anti-Cdc42 antibody or an anti-EGFP antibody. *B*, kinase activity of different PAK6 mutants. HeLa cells were transfected with pcDNA3-EGFP expression vector containing 40 or 80 ng of either PAK6wt, PAK1wt, or PAK6 (H20L, H23L, K436A; H20L, H23L; and H20L, H23L, S531N) cDNAs. PAK proteins were immunoprecipitated and incubated with Cdc42 loaded with either GDP or GTPyS; a kinase assay was performed in the presence of histone H3/H4, and the result was analyzed by autoradiography. *C*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40- β -gal, 40 ng of pSV-hAR, and 40 or 80 ng of pcDNA3-EGFP-PAK6 constructs. Total amount of DNA was normalized with pcDNA3. 18 h after transfection cells were stimulated with 10 nM R1881 for another 24 h and cell extracts prepared, and luciferase activity was monitored. The bar graph shown represents the mean \pm S.E. % inhibition of AR-mediated transcription by different PAK6 mutants observed in three independent experiments.

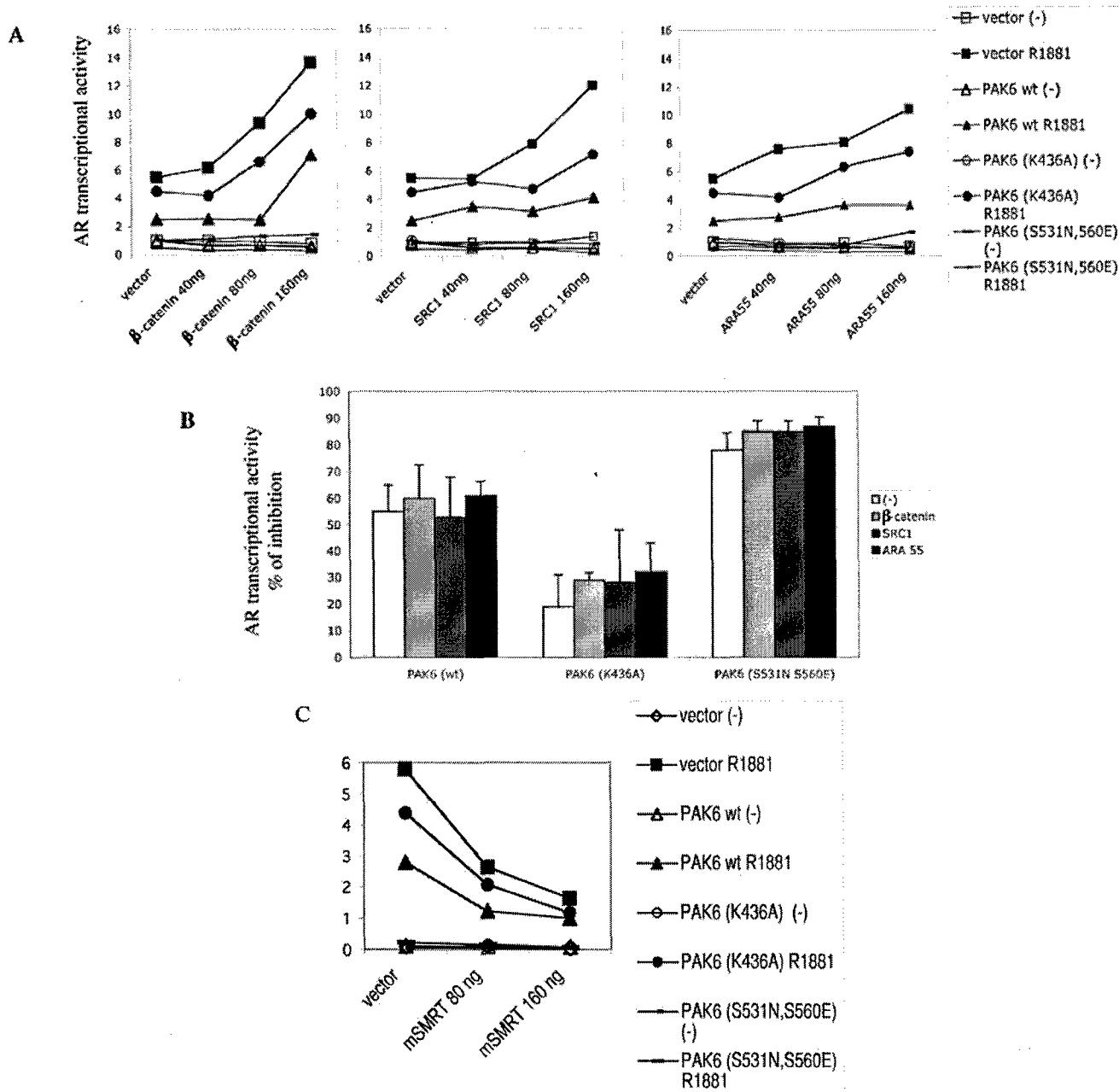


FIG. 5. PAK6 inhibits AR-mediated transcription in the presence of AR coactivators. *A*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40- β -gal, 40 ng of pSV-HAR, 40 ng of pcDNA3-EGFP-PAK6 construct, and increasing amounts of pcDNA3- β -catenin, pcDNA3-SRC1, or pSG5-ARA55 constructs (40, 80, and 160 ng). The amount of DNA was normalized with pcDNA3. 18 h after transfection, cells were stimulated with 10 nM of R1881 (black symbols) or left untreated (open symbols) for another 24 h. Luciferase activity was determined, reported as relative light units. Results shown are representative of three similar experiments. *B*, the bar graph shown represents the mean \pm S.E. % inhibition of AR-mediated transcription by PAK6 in presence of AR coactivators. *C*, CV-1 cells were transiently transfected in 12-well plates with 400 ng of pMMTV-Luc, 100 ng of pSV40- β -gal, 40 ng of pSV-HAR, 40 ng of pcDNA3-EGFP-PAK6 construct, and increasing amounts of pCMX-mSMRTa construct (80 and 160 ng). The amount of DNA was normalized with pcDNA3. 18 h after transfection, cells were stimulated with 10 nM R1881 (black symbols) or left untreated (open symbols) for another 24 h. Luciferase activity was determined, reported as relative light units. Results shown are representative of four similar experiments.

with either GDP or GTP γ S (Fig. 4A). PAK6wt was able to pull down Cdc42 loaded with GTP γ S, whereas the H20L,H23L mutation abrogated binding of Cdc42 to PAK6. In order to verify that the mutation H20L,H23L did not modify the kinase activity of PAK6, PAK6wt and CRIB mutants were transiently expressed in HeLa cells, and equal amounts of PAK6 were immunopurified from cell lysates using EGFP antibody. An *in vitro* kinase assay using histone H3/H4 was performed in the presence of Cdc42 loaded with either GDP or GTP γ S. As shown in Fig. 4B, PAK6(H20L,H23L) exhibited the same relative level

of exogenous kinase and autophosphorylation activity as did PAK6wt, whereas PAK6(H20L,H23L,K436A) exhibited no kinase activity at all. The mutant H20L,H23L,S531N displayed a strong exogenous kinase and autophosphorylation activity.

We next analyzed the ability of the non-Cdc42-binding H20L,H23L PAK6 mutants to suppress AR-mediated transcriptional activity. CV1 cells were transfected with pSVAR, MMTVpA3-Luc, PAK6wt, and PAK6 mutants. Mutation of H20L,H23L did not significantly modify the inhibitory effect of wt and kinase-active (S531N) forms of PAK6 (Fig. 4C). These

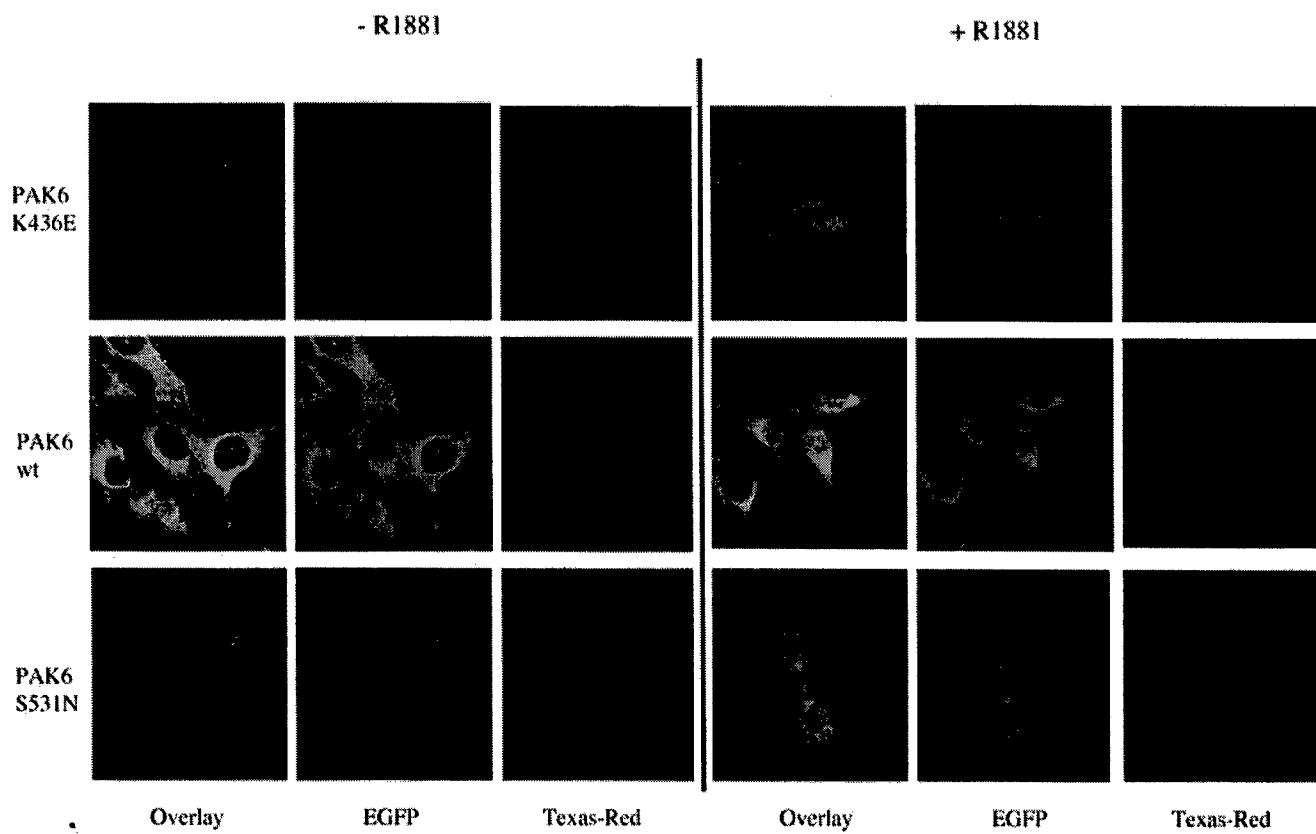


FIG. 6. PAK6 inhibits AR nuclear translocation. HeLa cells were transfected with wt or mutated EGFP-PAK6 and AR expression constructs and were cultured in the presence or absence of R1881 (10 nM). AR protein was detected with a polyclonal anti-AR antibody and revealed by Texas Red-conjugated secondary antibody. Pictures of cells were taken with confocal microscopy.

results demonstrate that PAK6-induced inhibition of AR-mediated transcription is independent of Cdc42 binding.

PAK6 Inhibits AR-mediated Transcription Enhanced by AR Coactivators and Inhibited with AR Corepressors—AR transcriptional activity can be enhanced by the presence of AR coactivators, including β -catenin, SRC1, ARA55, p300, and Tip60 α , which have been shown to bind to distinct regions of the AR and increase AR-mediated transcription. We sought to determine whether PAK6wt and PAK6 mutants were able to inhibit to the same extent AR-mediated transcription in the presence of these differentially binding AR coactivators. CV1 cells were transfected with pSVAR, MMTVpA3-Luc, PAK6wt, and PAK6 mutants and serial concentrations of either β -catenin, SRC1, or ARA55. The coactivators β -catenin, SRC1, and ARA55 all increased AR-mediated transcription in a dose-dependent manner (Fig. 5A). At a concentration of 160 ng, β -catenin, SRC1, and ARA increased AR-mediated transcription by 2.3-, 2-, and 1.6-fold, respectively. In the presence of the highly active PAK6(S531N,S560E) mutant, these coactivators were not able to increase significantly the transcriptional activity of AR. In contrast, in the presence of PAK6wt or K436A, these coactivators all increased AR transcriptional activity. As shown in Fig. 5B, the inhibitory effect of PAK6wt on AR-mediated transcription was 55% in the absence of the coactivator and 60, 53, and 61% in the presence of β -catenin, SRC1, or ARA55, respectively. The inhibitory effect of PAK6(K436A) on AR-mediated transcription was 20% in the absence of the coactivator and 29, 28, and 32% in the presence of β -catenin, SRC1, or ARA55, respectively. The inhibitory effect of the dominant active mutant PAK6(S531N,S560E) on AR-mediated transcription was 80% in the absence of the coactivator and 85, 85, and 87% in the presence of β -catenin, SRC1, or ARA55, respectively. PAK6 also inhibited AR-mediated transcription in the

presence of the coactivators p300 and Tip60 α (data not shown). In addition, as shown in Fig. 5C, PAK6 increased the inhibition of AR signaling seen with the AR corepressor SMRT. These results show that the inhibitory effect of PAK6 on AR-mediated transcription is dominant over the presence of the AR activating cofactors β -catenin, SRC1, ARA55, p300, and Tip60 α and are observed in the presence of a corepressor, SMRT, suggesting that PAK6 may act to directly modulate AR function.

Nuclear Translocation of the AR in Response to Androgen Is Inhibited by Kinase-active Mutants of PAK6—To assess the biological function of PAK6 kinase activity, we examined the subcellular localization of the AR after stimulation with androgen in the presence of PAK6 and PAK6 mutants (K436A and S531N) by immunofluorescence. As shown in Fig. 6, in the absence of R1881 the AR localized to the cytoplasm and the nucleus. In control (GFP-transfected) cells, R1881 induced a total nuclear accumulation of the AR, as reported previously (not shown). Similarly, in the presence of the kinase-inactive PAK6(K436A), treatment with R1881 resulted in the total accumulation of the AR in the nucleus. In contrast, upon co-expression of the AR with PAK6wt or highly active PAK6(S531N), R1881-induced translocation of the AR was partially inhibited, with a good deal of receptor remaining in the cytoplasm. These results suggest that PAK6 kinase activity negatively modulates steroid-induced AR translocation into the nucleus.

PAK6 Phosphorylates the AR in the DBD Domain—In order to further investigate the biochemical basis of PAK6-induced inhibition of AR-mediated signaling, we sought to determine whether PAK6 could phosphorylate the AR. To that end, we assessed the ability of PAK6 to phosphorylate a series of recombinant AR proteins (Fig. 7A). Highly active PAK6(S531N) was immunoprecipitated from HeLa cell lysates and incubated

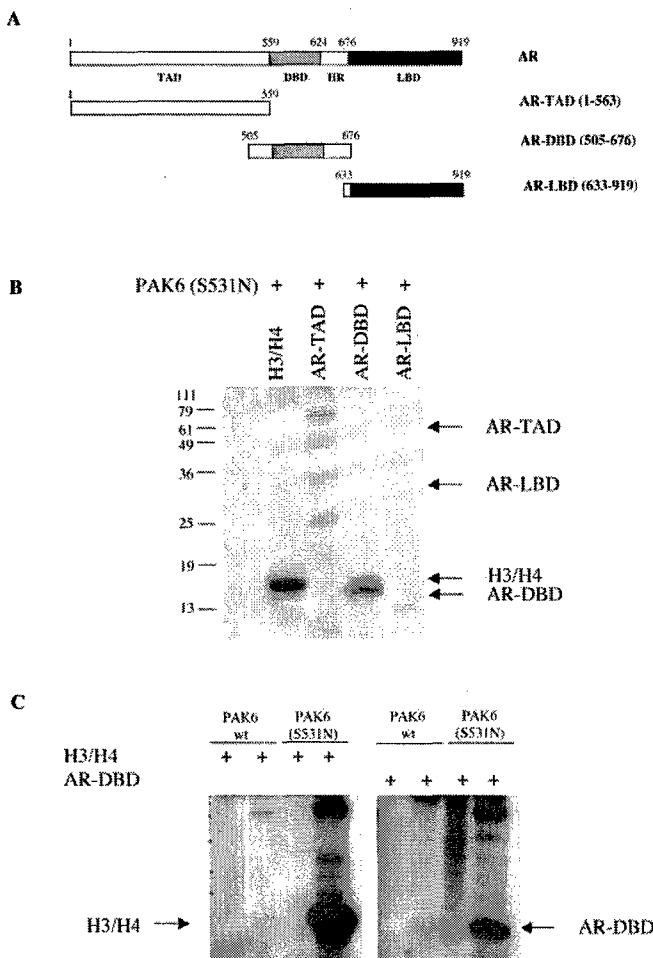


FIG. 7. PAK6 phosphorylates the AR-DBD domain. *A*, schematic representation of AR proteins. AR-TAD, AR-DBD, and AR-LBD were expressed in *Escherichia coli* and used as substrates in kinase assays. TAD, DBD, LBD, and hinge region (HR). *B*, immunoprecipitated PAK6(S531N) was used in a kinase assay with 1 μ g of H3/H4 or 1 μ g of the different domains of the AR. Phosphorylation of H3/H4 and the AR fragments was detected by autoradiography. *C*, PAK6wt or PAK6(S531N) was immunoprecipitated from HeLa cells and incubated with 1 μ g of H3/H4 or DBD-AR purified from *E. coli*, and a kinase assay was performed. Phosphorylation of H3/H4 and the DBD fragments were detected by autoradiography.

with recombinant AR proteins in an *in vitro* kinase assay. PAK6(S531N) strongly phosphorylated a construct containing the DBD of the AR (Fig. 7*B*). In contrast, no phosphorylation of the AR TAD or the AR LBD could be detected. PAK6wt also phosphorylated the DBD-containing fragment, although phosphorylation was weaker than with PAK6(S531N) (Fig. 7*C*). These results suggest that PAK6 could modify AR activity through the direct phosphorylation of the DNA-binding element, thereby potentially preventing transcriptional regulation from taking place.

PAK6 Protein Expression in Prostate Cancer Cell Lines—To better understand the potential biological role(s) of PAK6, we analyzed the expression of PAK6 protein in different cancer cell lines. The antiphospho-PAK6 (Ser(P)-560) antibody was used to analyze PAK6 in prostate cancer cell lines DU145, PC-3zj, PC-3, ARCap, PC3MM2, LNCaP, and LAPC4, breast cancer cell line MCF7, and the uterine cancer cell line HeLa (Fig. 8). As shown in Fig. 2*D*, this antibody was able to detect active versions (PAK6wt and PAK6(S531N)) but did not recognize the inactive PAK6(K436A) mutant. Phospho-PAK6 was detected most abundantly in the LAPC4 cell line. High expression/ac-

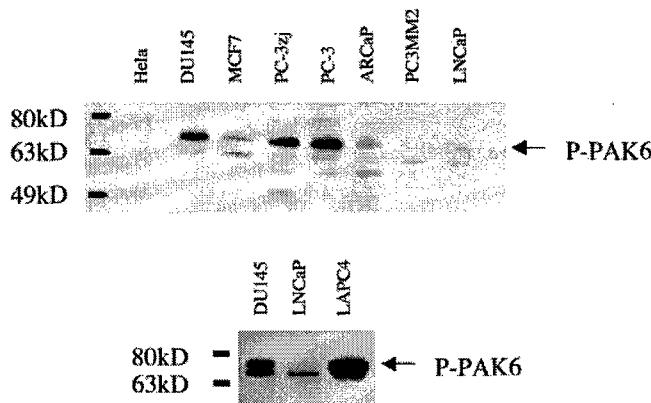


FIG. 8. PAK6 protein expression in human cancer cell lines. *A*, Western blot was performed on equal protein amounts of lysates from prostate cancer cell lines (DU145, PC-3zj, PC3, Arcap, PC3MM2, Lncap, and LapC4), breast cancer cell lines (MCF7), and uterus cancer cells line (HeLa) by using antibody recognizing PAK6 protein phosphorylated on serine 560. *B*, Western blot was performed on equal protein amounts of lysates from DU145, LNCaP, and LAPC4 by using antibody recognizing PAK6 protein phosphorylated on serine 560.

tivity levels were also detected in the DU145, PC-3, and PC-3zj lines. MCF7 and ARCap expressed only a small amount of phospho-PAK6, and no expression was detected in HeLa, LNCaP, and PC3-mm2 cells. These results indicate that the expression and/or activity level of PAK6 in prostate cell lines is highly variable and suggest that PAK6 could be involved in differential regulation of AR signaling in these cells.

DISCUSSION

In this study, we have investigated the mechanism by which the protein kinase PAK6 inhibits AR-mediated transcription. In order to better characterize the regulation of this serine/threonine kinase, we compared the kinase activity of PAK6 to PAK1 and PAK4, two well characterized members of the group I and group II PAK protein family, respectively. It is well established that the binding of activated Rac and Cdc42 GTPases to PAK1 markedly stimulates its kinase activity, both autophosphorylation and activity toward exogenously supplied substrates (3, 33, 34). In contrast, Rac or Cdc42 GTPase binding to PAK4 does not stimulate the already rather high basal kinase activity (4). We found that, like PAK4, PAK6 exhibits a constitutive kinase activity that is not increased by active Cdc42 (or Rac, not shown) GTPase (Figs. 1*A* and 2*D*). These findings are in accordance to BLAST comparisons showing that, like PAK4 and PAK5, PAK6 lacks the autoinhibitory domain adjacent to the CRIB domain in the group I PAKs whose release by GTPase binding accounts for GTPase-dependent kinase activation.

Whereas two prior studies (7, 8) have described the ability of PAK6 to suppress transcriptional signaling downstream of the androgen and estrogen nuclear receptors, the mechanism(s) underlying this effect have not been determined. We showed that mutation of PAK6 (PAK6(S531N)) to increase its kinase activity relative to the wild type protein dramatically increased its inhibitory effect on AR-mediated transcription (Fig. 3). Conversely, abrogating PAK6 kinase activity (PAK6(K436A)) considerably decreased its inhibitory effect on AR transcriptional activity. These results demonstrate that PAK6 kinase activity is involved in inhibition of AR-mediated transcription. It is also interesting to notice that the kinase-dead mutant of PAK6 (K436A) is still able to inhibit ~20% of AR transcriptional activity (Figs. 3 and 4). This result suggests that PAK6 is also able to suppress the transcriptional activity of the AR by a phosphorylation-independent mechanism. Neither PAK1 nor PAK4 was effective at inhibiting AR signaling, suggesting a mechanism specific to PAK6 is operative. Both prior studies

have indicated a physical interaction of PAK6 with the AR, including a region between the hinge and LBD. By BLAST comparison we have found that PAK6 possesses a unique region localized between amino acids 289 and 307. This domain is homologous to the Ω loop of Rbp8, one of the subunits of RNA polymerase (35, 36). We speculate that this domain could also be important for the PAK6 and AR interaction.

Because the regulation of transcription by the hormone-bound AR requires relocalization of the receptor to the nucleus, we considered the possibility that the binding of GTPase to PAK6 might play some role in this translocation process. We mutated conserved residues within the PAK6 CRIB domain known to be critical for GTPase binding (H20L, H23L). We established that this mutation impeded the binding of Cdc42 to PAK6 without affecting the inhibitory action of PAK6 on AR transcriptional activity (Fig. 4). These results demonstrate that Cdc42 binding is not required for PAK6-mediated inhibition of AR transcriptional activity, and also suggest that PAK6 activity is not modulated by GTPase-dependent relocalization within the cell. It is possible that the interaction of PAK6 with Cdc42 modulates an as yet unknown aspect of PAK6 function that is independent of the observed regulation of AR transcriptional activity.

The inhibition of AR signaling by PAK6 might be the result of a direct effect on the AR or an indirect effect on other AR-associated proteins. To address this issue, we studied the effect of PAK6 on AR signaling in the presence of different AR-associated coactivator and corepressor molecules, β -catenin, SRC1, ARA55, p300, Tip60 α , and SMRT, all of which have been reported to increase or decrease AR transcriptional activity (Fig. 5). The presence of these coregulators did not modify the relative inhibitory effect of PAK6 and PAK6 mutants (K436A and S531N) on AR transcriptional activity. These results indicate that the mechanism for AR inhibition by PAK6 does not involve an indirect effect on the AR coactivators, β -catenin, SRC1, and ARA55, and that inhibition occurs through a common component utilized by each coactivator or corepressor (i.e. the receptor itself).

Given the fact that PAK6 inhibition of AR-mediated transcription is dependent on its kinase activity and is independent of AR coactivators, we examined the effects of PAK6 on AR nuclear translocation (Fig. 6). Kinase-active versions of PAK6 partially blocked steroid-induced nuclear translocation of the receptor. The lack of nuclear AR would tend to inhibit the transcriptional activity of the receptor. Interestingly, we did not observe steroid-induced nuclear translocation of PAK6, as had been originally reported by Yang *et al.* (7). We cannot rule out that this difference is because of cell type differences or to the use of GFP-tagged PAK6 in the current studies.

We also tested whether PAK6 was able to directly phosphorylate the AR (Fig. 7). We found that wild type and the highly active PAK6 mutant (S531N) were able to phosphorylate a fragment of the AR including the DBD and the hinge domains *in vitro*. It has been reported that PAK6 binds to a region between the hinge region and LBD of the AR. This suggests a scenario in which the *in vivo* binding of PAK6 to this region positions PAK6 to phosphorylate the AR and regulate activity of the nearby DBD domain. Because this region is important for DNA binding by the AR, phosphorylation in this domain by PAK6 could inhibit the binding of AR to DNA and, by this mechanism, suppress its transcriptional activity. There are several potential phosphorylation sites for PAK6 in the AR DBD domain. Of particular interest, Gioeli *et al.* (37) have reported that Ser-650 in the DBD is phosphorylated in response to epidermal growth factor receptor stimulation, which is known to recruit PAK (38). A Ser-650 \rightarrow Ala mutation has

been suggested previously by Zhou *et al.* (39) to regulate AR transactivation of the mouse mammary tumor virus promoter when suboptimal levels of steroid were used. This phosphorylation site in the hinge region is conserved in many of the steroid receptors (40). The identification of the relevant PAK6 phosphorylation sites in the AR and the investigation of this potential regulatory mechanism will be a subject of future studies.

We have shown by using PAK6 mutant proteins of varying activity that serine 560 phosphorylation reflects the kinase activity of PAK6. We used a phosphoserine 560 PAK6 antibody to examine in prostate cancer cell line the expression of active PAK6 (Fig. 7). We found that expression of phosphorylated PAK6 in these cell lines is quite variable. Interestingly, we did not detect phospho-PAK6 expression in the androgen-sensitive cell line LNCaP, but we observed a strong expression of phospho-PAK6 in the androgen-insensitive cell lines DU145 and PC3. These results suggest that PAK6 up-regulation might account for the development and/or maintenance of androgen independence, which is known to be associated with more aggressive tumors. In this regard, the activity common to the PAK family members of modulating cell motility and dynamics may come into play for PAK6 as well. It will be of interest in future studies using a larger sampling of prostate cancer cell lines and tumor tissues to assess the relationship of PAK6 activity to AR phosphorylation state, responsiveness to androgens, and the growth rates and metastatic capabilities of these cancer cells.

In conclusion, this study demonstrates that PAK6 inhibition of AR-mediated transcription is dependent on the kinase activity of PAK6 and is specific to this PAK isoform. PAK6 may act by multiple mechanisms to antagonize transcriptional activity of the AR. Most importantly, we present evidence that expression of active PAK6 in cancer prostate cells is variable but may correlate with androgen sensitivity of the lines. These data suggest that modulation of PAK6 activity may be an important mechanism contributing to the regulation of AR signaling in various forms of prostate cancer.

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Wnt3a Growth Factor Induces Androgen Receptor-Mediated Transcription and Enhances Cell Growth in Human Prostate Cancer Cells

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ABSTRACT

The Wnt signaling pathway plays a critical role in embryogenesis and tumorigenesis. However, biological roles of Wnt growth factors have not been fully characterized in prostate development and the pathogenesis of prostate cancer. In this study, we used Wnt3a-conditioned medium (Wnt3a-CM) and purified Wnt3a proteins to investigate whether there is a direct effect of Wnt3a on androgen receptor (AR)-mediated transcription and to determine its role in the growth of prostate cancer cells. We demonstrated that Wnt3a-CM either induces AR activity in the absence of androgens or enhances AR activity in the presence of low concentrations of androgens, whereas purified Wnt3a showed a pronounced effect in the presence of low concentrations of ligands. We also showed that Wnt3a-CM and the purified Wnt3a enhance the level of cytosolic and nuclear β -catenin, suggesting an involvement of β -catenin in this regulation. Moreover, treatment of LNCaP cells with Wnt3a-CM and purified Wnt3a significantly enhances cell growth in the absence of androgens. Our findings demonstrate that Wnt3a plays an important role in androgen-mediated transcription and cell growth. These results suggest a novel mechanism for the progression of prostate cancer.

INTRODUCTION

Prostate cancer is the most common malignancy in men and the second leading cause of cancer deaths in the United States (1). The androgen signaling pathway, which is mainly mediated through the androgen receptor (AR), is important for the normal and neoplastic development of prostate cells (2, 3). Androgen ablation is an effective treatment for the majority of patients with advanced prostate cancer (4). However, most of the patients develop androgen-insensitive prostate cancer within 2 years, for which there is currently no effective treatment. Multiple mechanisms by which prostate cancer cells progress to androgen-insensitive stages have been proposed (3, 5). Recently, several lines of evidence have led to an increased interest in defining the possible role of Wnt signaling in the development and progression of prostate cancer [please see the review by Chesire and Isaacs (6)].

The Wnt ligands, of which there are more than 19 closely related but distinct secreted cysteine-rich glycoproteins, have been characterized according to their roles in early development and tumorigenesis.⁴ Evidence from recent studies suggests critical roles for the Wnt ligands in controlling cell proliferation, adhesion, survival, movement, and polarity (7, 8). Receptors for the Wnt proteins are members of the Frizzled family. In vertebrates, Wnt proteins activate different intracellular signaling cascades either through the “canonical” or “non-canonical” pathways (9). In the canonical pathway, secreted Wnt ligands bind to Frizzled and regulate the stability of β -catenin, a

key component of Wnt signaling. In the absence of a Wnt signal, β -catenin is constitutively down-regulated by the multicomponent destruction complex containing glycogen synthase kinase 3 β , axin, and APC, which promotes phosphorylation on the serine and threonine residues in the NH₂-terminal region of β -catenin and thereby targets it for degradation through the ubiquitin proteasome pathway (10). Wnt signaling inhibits this process, which leads to accumulation of β -catenin in the nucleus and promotes the formation of transcriptionally active complexes with lymphoid enhancer-binding factor (LEF)/T-cell factor (TCF) transcription factors (11, 12).

Wnt signaling and its key component, β -catenin, have been implicated in human malignancy (13, 14). The link between stabilized β -catenin and tumorigenesis was considerably strengthened by discoveries of mutations in the destruction complex and in β -catenin itself in a variety of human tumors (15). Loss of control of intracellular β -catenin levels through mutation in β -catenin itself and/or other components of the protein degradation complex has been reported in prostate cancer samples (16, 17). However, only a small proportion of prostate cancer samples possessed these mutations, suggesting that other possible mechanisms may be involved in the regulation. It has been shown that loss of E-cadherin can result in an increase of the cellular β -catenin in prostate cancer cells (18). Overexpression of E-cadherin in E-cadherin-negative tumor cells decreases cellular β -catenin levels and reduces AR-mediated transcription (19).

A protein–protein interaction between the AR and β -catenin has been identified (19–21). Through this interaction, β -catenin acts as an AR coactivator, augmenting AR-mediated transcription (19). These data provided an additional line of evidence linking Wnt/ β -catenin to the androgen signaling pathway in the growth and progression of prostate cancer.

Potential roles for Wnt in tumorigenesis were suggested previously (22, 23). However, the molecular mechanisms by which Wnt signaling regulates the growth and progression of tumor cells are unclear. Knowledge regarding Wnt signaling in the pathogenesis of prostate cancer is lacking. In this study, we examine the role of Wnt 3A in the regulation of androgen signaling in prostate cancer cells. Intriguingly, we demonstrated that Wnt3a induces AR-mediated transcription and cell growth in a ligand-independent manner. These findings provide the first line of evidence that the Wnt growth factor can regulate and interact with the androgen signaling pathway in prostate cancer cells, which suggests a novel mechanism for the development of androgen-insensitive prostate cancer.

MATERIALS AND METHODS

Cell Culture and Conditioned Medium Production. The monkey kidney cell line CV-1 and human prostate cell lines DU145 and PC3 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS; HyClone, Denver, CO). An AR-positive prostate cancer cell line, LNCaP, was maintained in T medium (Invitrogen, Carlsbad, CA) with 5% FCS. Wnt3a-conditioned medium (Wnt3a-CM) and L cell control medium (L-CM) were prepared as described previously (24). Briefly, mouse L cells stably transfected with a Wnt3a cDNA driven by the rat phosphoglycerokinase gene promoter were cultured in DMEM supplemented with 10% charcoal-stripped FCS (CS-FCS) for 4 days. The Wnt3a-CM was then harvested, centrifuged at 1,000 \times g for 15 minutes, and filtered using 0.45 μ m cellulose

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⁴ <http://www.stanford.edu/~rnuusse/wntwindow.html>.

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acetate bottle top filters (Corning, New York, NY). L-CM was prepared under the same conditions from L cells stably transfected with the pGKneo vector alone (24).

Plasmid Construction. The pGL3-OT and pGL3-OF constructs were the gifts of Dr. Bert Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD). The pcDNA-TCF-1 construct was provided by Dr. H. C. Clevers (Center for Biomedical Genetics, Utrecht, the Netherlands). The pPSA7kb-luc plasmid was obtained from Dr. Jan Trapman (Department of Pathology, Erasmus University, Rotterdam, The Netherlands) (25). A cytomegalovirus-driven β -galactosidase (β -gal) reporter was generated by cloning the lacZ gene into the pcDNA3 vector (19). A double-stranded oligonucleotide corresponding to the human AR cDNA sequence (5'-GGT-GTCAGTATGGAGCTCTCA-3', amino acids 568–575) was synthesized and cloned into the pBS/U6 vector, provided by Dr. Yang Shi (Harvard Medical School, Boston, MA) to make the short hairpin RNA (shRNA) construct (26).

Transfection, Luciferase, and β -gal Assays. Transient transfections were carried out using LipofectAMINE 2000 (Invitrogen). Cells were incubated with Wnt3a-CM or L-CM in the presence or absence of dihydrotestosterone (DHT) 24 hours after transfection. After an 18- to 24-hour incubation, cells were harvested, and the luciferase and β -gal activities were measured. The relative light units (RLU) from individual transfections were normalized using β -gal activity in the same samples. Individual transfection experiments were done in triplicate, and the results are reported as mean RLU/ β -gal (\pm SD).

Preparation of Cell Fractions. LNCaP cells treated with Wnt3a-CM or the control L-CM were grown to confluence in 6-well plates, washed once with PBS, and harvested by scraping. Cells were then centrifuged at 750 \times g for 2 minutes, resuspended in a hypotonic buffer [10 mmol/L Tris-HCl (pH 7.8), 10 mmol/L KCl, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μ g/mL aprotinin, and 10 μ g/mL leupeptin] and incubated on ice for 10 minutes. The cells were lysed by Dounce homogenization and then centrifuged at 2,000 \times g for 30 minutes to pellet unlysed cells and nuclei. The cytosolic fraction was obtained by further fractionation at 100,000 \times g for 1 hour.

Northern Blotting. Total RNA from LNCaP cells treated with Wnt3a-CM or L-CM in the presence or absence of DHT was isolated using a RNAwiz kit (Ambion, Austin, TX). Six micrograms of RNA were fractionated on a 1% agarose-formaldehyde gel, transferred to Hybond-N nylon membranes (Amersham Biosciences, Piscataway, NJ), and hybridized with a DNA fragment (amino acids 1–261) derived from the human prostate-specific antigen (PSA) gene. Hybridization was performed overnight at 65°C in 0.5 mol/L sodium phosphate (pH 7.2), 1% bovine serum albumin, and 7% SDS. The blots were stripped and rehybridized with a human glyceraldehyde-3-phosphate dehydrogenase probe (27).

Immunoprecipitation and Western Blotting. Coimmunoprecipitation assays were carried out essentially as described previously (28). Proteins were eluted by boiling in SDS-sample buffer, resolved by 10% SDS-PAGE, and transferred onto a nitrocellulose membrane. The membranes were then probed with a 1:500 dilution of a polyclonal antibody against the NH₂ terminus of AR (Upstate, Charlottesville, VA) or an anti- β -catenin monoclonal antibody. Proteins were detected using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL). The anti-Wnt3a polyclonal antibody was generated and used in the study.

Immunofluorescence. Cells were cultured in 8-well Lab Tek chambered cover slides (Nalge Nunc International, Naperville, IL), fixed in 4% paraformaldehyde for 15 minutes, and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were then incubated with anti- β -catenin monoclonal antibody (Signaling Transduction Laboratories, Lexington, KY) for 1 hours and labeled with anti-mouse 594 Alexa secondary antibody (Molecular Probes, Eugene, OR). The nuclei were counterstained with 10 μ g/mL Hoechst (Molecular Probes). Samples were analyzed with a Zeiss LSM confocal laser scanning microscope.

Cell Proliferation and Colony Formation. Approximately 2,000 cells per well were plated and cultured in the presence of either Wnt3a-CM or L-CM and then harvested at different time points. Proliferation assays were carried out using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonylphenyl)-2H-tetrazolium (MTS) kit (Promega, Madison, WI). Cell numbers were determined by absorbance at 490 nm as suggested by the manufacturer. For colony formation assay, LNCaP cells were plated in 6-well plates (500–1,000 cells per well) for 24 hours and then maintained in Wnt3a-CM or L-CM or in DMEM containing purified Wnt3a proteins for 10 to 12 days. The

cells were stained with crystal violet (Sigma, St. Louis, MO), and colonies containing more than 50 cells were counted. Colony assays were performed a minimum of three times, and the results are reported as a mean of three experiments.

Purification of Wnt3a. Purified Wnt3a proteins were isolated as described previously (29). A detailed protocol can also be found on-line.⁴

RESULTS

Wnt3a-CM Enhances AR-Mediated Transcription in a Ligand-Independent Manner. The Wnt pathway has been implicated in the growth and differentiation of various tissues and organs. Recent data showing that β -catenin, a key player in the Wnt pathway, interacts with the AR provided a direct link between Wnt signaling and the pathogenesis of prostate cancer (19–21). In this study, we first used Wnt3a-CM to directly investigate the role of Wnt signaling in prostate cancer cells. Using a specific antibody, we verified the expression of Wnt3a in the Wnt3a-CM prepared from the mouse L cells (Fig. 1A). We found that treatment with Wnt3a-CM of both mouse L and DU145 cells increased the level of cytosolic β -catenin (Fig. 1B). Moreover, the Wnt3a-CM induced β -catenin-mediated TCF-1 transcription (Fig. 1C). These results are consistent with a previous report and confirmed the properties of the Wnt3a-CM (24).

To evaluate the effect of Wnt3a on AR-mediated transcription, we transfected a luciferase reporter driven by the 7-kb PSA gene promoter, an AR-regulated target gene (30), into LNCaP cells. The cells were cultured in the presence or absence of DHT with or without Wnt3a-CM. As shown in Fig. 2A, Wnt3a-CM significantly increased endogenous AR-mediated transcription from the PSA promoter. Interestingly, cells treated with the Wnt3a-CM showed an approximately

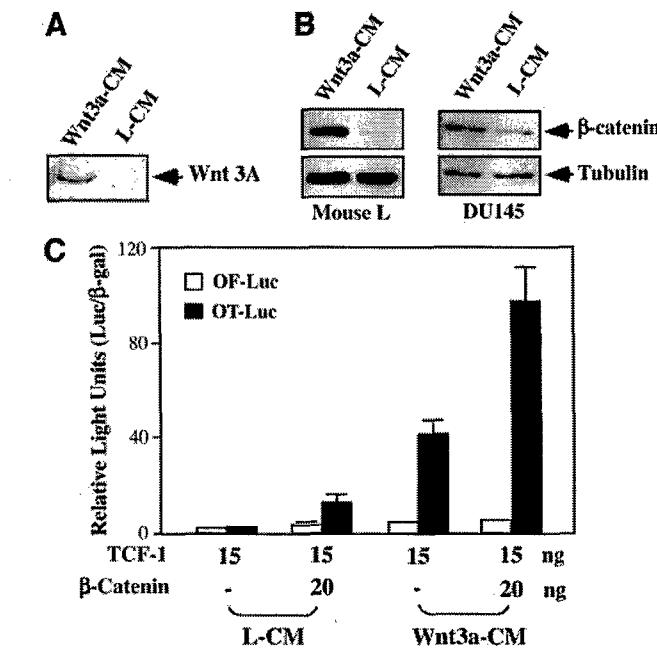


Fig. 1. Wnt3a-CM stabilizes β -catenin and stimulates TCF-mediated transcription. A. Ten microliters of Wnt3a-CM and control medium were analyzed by Western blot with a rabbit polyclonal antibody against Wnt3a. B. Mouse L and DU145 (a human prostate cancer cell line) cells were cultured with either Wnt3a-CM or L-CM for 20 hours. Whole cell lysates were prepared and analyzed by Western blot with an anti- β -catenin antibody. The samples were also probed with an anti-tubulin antibody to verify equal loading. C. CV-1 cells were transiently transfected with 100 ng of pGL3-OT (OT-Luc) or the inactive mutant pGL3-OF (OF-Luc), 25 ng of pcDNA3- β -gal (β -gal), and other plasmids as identified in the figure. The transfected cells were incubated in DMEM with 5% FCS for 24 hours, washed, and then cultured with either Wnt3a-CM or L-CM for another 24 hours. The cells were harvested, and the luciferase and β -gal activities were measured. Luciferase activity is reported as RLU (luciferase/ β -gal) and represented as mean \pm SD.

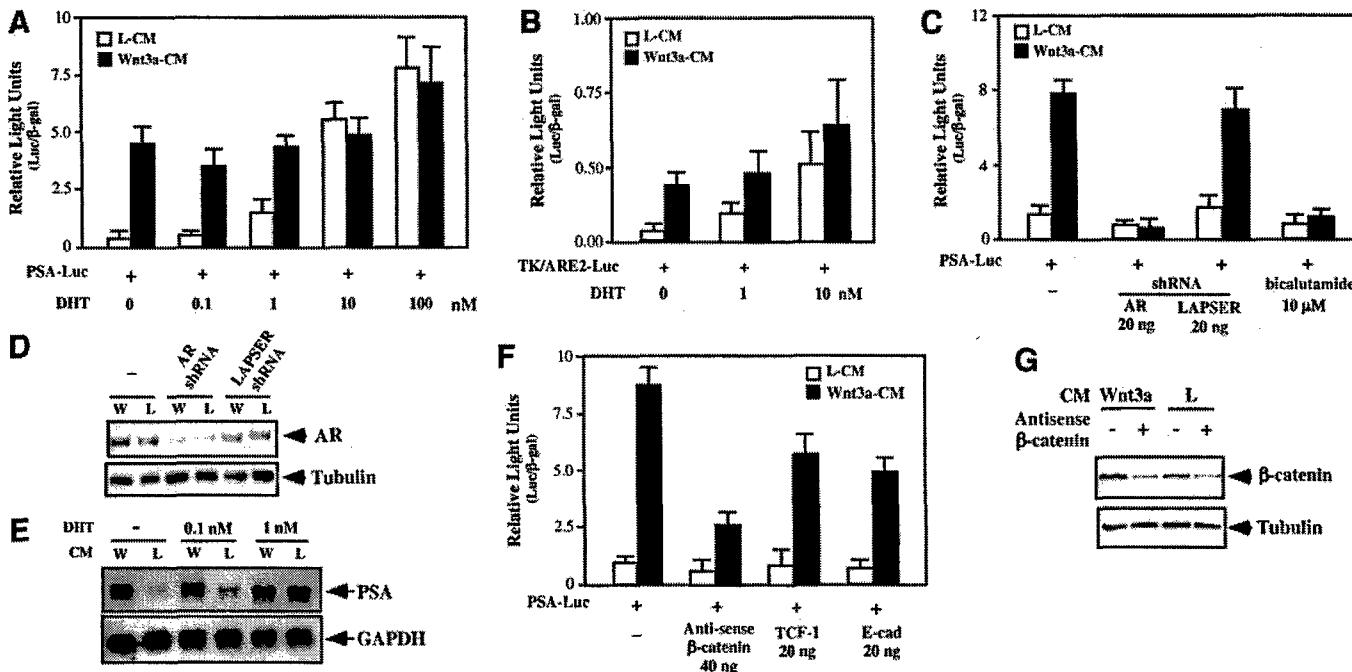


Fig. 2. Wnt3a-CM induces AR-mediated transcription. **A.** LNCaP cells were transiently transfected with 100 ng of PSA-Luc and 25 ng of pcDNA3- β -gal and incubated in T-medium with 5% CS-FCS for 16 hours. The cells were washed and incubated with Wnt3a-CM or L-CM in the presence of different amounts of DHT for another 24 hours. Luciferase and β -Gal activities were measured and reported as RLU. **B.** LNCaP cells were transfected with 100 ng of TK/ARE2-luc and 25 ng of pcDNA3- β -Gal and treated as described above. Luciferase and β -gal activities were measured. **C.** Transient transfections were carried out as described above. Twenty ng of AR shRNA plasmid or LAPSER shRNA vector (45), as the control, and other plasmids as marked in the figure were transfected into LNCaP cells. Cells were cultured with either Wnt3a-CM or L-CM. An AR antagonist, bicalutamide, was added into cells 24 hours after transfection. Luciferase and β -gal activities were measured. **D.** The cytosolic fractions were prepared from the above-mentioned LNCaP cells transfected with AR or LAPSER shRNA constructs and analyzed by Western blot with AR and tubulin antibodies. **E.** LNCaP cells were cultured with Wnt3a-CM or L-CM, in the absence of DHT or in the presence of 0.1 or 1 nmol/L DHT, for 24 hours. Total RNA was isolated. Six micrograms of RNA were analyzed by Northern blot with a PSA probe (amino acids 1–261) derived from the human PSA gene. A human glyceraldehyde-3-phosphate dehydrogenase probe (GAPDH; amino acids 104–168) was used to verify equal loading. **F.** LNCaP cells were transiently transfected with 100 ng of PSA-Luc, 25 ng of pcDNA3- β -gal, and 40 ng of antisense β -catenin vector or 20 ng of TCF-1 or E-cadherin expression vector. Transfected cells were incubated in T-medium with 5% CS-FCS for 24 hours and then treated as described in **A**. **G.** Cytosolic fractions were isolated from the above-mentioned cells and analyzed by Western blot with β -catenin and tubulin antibodies.

11-fold induction of AR activity compared with cells treated with the control medium in the absence of DHT. These results provide the first evidence that the Wnt growth factor can independently activate AR-mediated transcription. In addition, Wnt3a-CM also showed an induction of AR-mediated transcription in the presence of 0.1 and 1 nmol/L DHT.

To verify that induction of the PSA promoter by Wnt3a-CM is a specific effect, we repeated the experiments in LNCaP cells with a luciferase reporter driven by a minimal promoter with two androgen response elements (AREs). A similar induction of AR-mediated transcription was observed in the cells treated with Wnt3a-CM (Fig. 2B). To further ensure that the induction by Wnt3a-CM is directly through the AR protein, we tested the effect of Wnt3a-CM on cells that were cotransfected with a shRNA construct of AR to knock down the AR protein. As shown in Fig. 2C, reduction of AR protein expression can abolish the AR-mediated transcription. This was correlated with a decreased level of cytosolic AR proteins in the cells (Fig. 2D). In addition, an AR antagonist, bicalutamide, can also block the activity of AR in cells treated with Wnt3a-CM (Fig. 2C). Taken together, these data indicate that the effect of Wnt3A is mediated through AR.

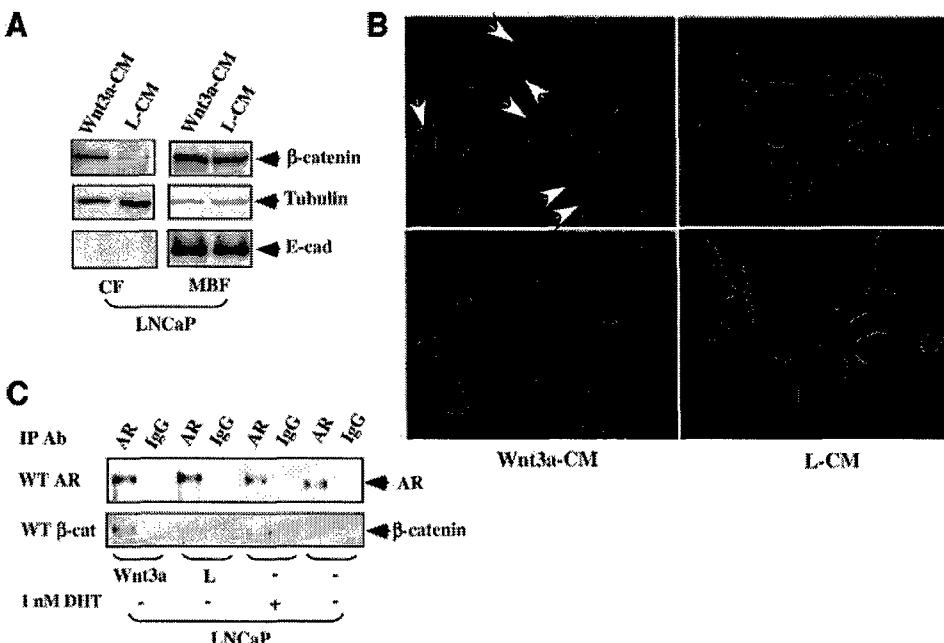
To evaluate the effect of Wnt3a-CM in a biologically relevant setting, we tested whether the conditioned medium regulates expression of the endogenous PSA gene. We measured transcripts of PSA in LNCaP cells treated with different amounts of DHT in the presence of Wnt3a-CM or control medium. As shown in Fig. 2E, Wnt3a-CM induces an approximately 5-fold increase in the expression of PSA in the absence of DHT. In addition, in the presence of 0.1 nmol/L DHT, the expression of PSA is about 2.5-fold higher in cells treated with Wnt3a-CM than in cells treated with L-CM (Fig. 2E). These results

provide an additional line of evidence that Wnt3a-CM can activate AR-mediated transcription. Taken together, we have demonstrated that Wnt3a-CM can activate AR-mediated transcription in the absence of ligand and augment AR activity in the presence of a low concentration of androgens.

Given that β -catenin is a key downstream effector of the Wnt pathway and acts as an AR coactivator, we further investigated whether β -catenin is involved in the Wnt3A-induced AR activity. We repeated the transient transfection experiments with an antisense construct of β -catenin. As observed previously (19), it specially reduces the level of cellular β -catenin proteins (Fig. 2G), and the induction of AR activity by Wnt3a-CM (Fig. 2F). It has been shown that overexpression of TCF/LEF and E-cadherin can compete for β -catenin binding to AR and reduce AR-mediated transactivation (31, 32). We therefore tested whether coexpression of TCF-1 and E-cadherin can affect the induction of AR activity mediated by Wnt3a-CM. As shown in Fig. 2F, PSA promoter/reporter activities were reduced approximately 35% to 45%, relative to the controls, in cells transfected with the TCF-1 and E-cadherin expression vectors. These data suggest an involvement of β -catenin in the induction of AR activity mediated by Wnt3a-CM.

Wnt3a-CM Increases the Level of Cytosolic and Nuclear β -Catenin in Prostate Cancer Cells. Wnt3a-CM has been shown to increase accumulation of cytosolic free β -catenin (24). Cotransfections of β -catenin antisense and TCF-1 and E-cadherin constructs suggested that β -catenin is involved in Wnt3a-CM-mediated AR activity. To evaluate whether Wnt3a-CM affects the cytosolic pool of β -catenin in LNCaP cells, we examined the levels of β -catenin in the different cellular fractions prepared from cells treated with the

Fig. 3. Wnt3a-CM enhances the level of cytosolic and nuclear β -catenin. A. LNCaP cells were cultured in Wnt3a-CM or L-CM for 24 hours and then harvested. A cytosolic fraction (CF) and membrane-associated fraction (MBF) were prepared and analyzed by Western blotting (see Materials and Methods). The expression of E-cadherin was examined to evaluate the purity of the above-mentioned fractions. **B.** LNCaP cells were cultured in Wnt3a-CM or L-CM for 24 hours, fixed with 4% paraformaldehyde for 15 minutes, and permeabilized with 0.2% Triton X-100 for 10 minutes. Cells were stained with the anti- β -catenin antibody followed by a secondary antibody conjugated with rhodamine (red). The nuclei were counterstained with Hoechst, and the ones with a high level of β -catenin are marked with arrows. **C.** Equal amounts of nuclear fractions isolated from LNCaP cells incubated with Wnt3a-CM (Wnt3a) or L-CM (L) were subjected to immunoprecipitation with normal mouse IgG or anti-AR monoclonal antibody. The nuclear fractions isolated from LNCaP cells cultured in the presence or absence of 1 nmol/L DHT were also included in experiments as controls. The precipitated fractions were then resolved by SDS-PAGE and analyzed by Western blot using anti- β -catenin or anti-AR antibodies (WT).



Wnt3a-CM and control medium. The cytosolic fraction and the membrane-associated fraction were prepared, representing the free cytosolic pool and membrane bound β -catenin, respectively (33). As shown in Fig. 3A, there was no significant change in the amount of β -catenin protein in the membrane-associated fraction isolated from the cells treated with Wnt3a-CM in comparison with untreated cells. However, there was a significant increase of cytosolic β -catenin in the cells treated with Wnt3a-CM compared with the controls. In addition, the level of tubulin, as a control, was similar in cells treated with Wnt3a-CM and L-CM in both the cytosolic fraction and membrane-associated fractions. The results indicate that Wnt3a-CM increased the levels of cytosolic β -catenin in LNCaP cells.

Next, we examined the effects of Wnt3a-CM on the cellular localization of β -catenin in LNCaP cells by immunofluorescence staining. As shown in Fig. 3B, clear cell membrane staining with the β -catenin antibody was observed in LNCaP cells. However, there is also an increase in nuclear β -catenin in cells treated with Wnt3a-CM. These data are consistent with the results from the Western blot (Fig. 3A) and suggest that Wnt3a-CM can stimulate nuclear translocation of β -catenin.

It has been shown that β -catenin forms a protein complex with AR and enhances AR-mediated transcription in LNCaP cells. Given that Wnt3a-CM enhances cytosolic free β -catenin and nuclear translocation of β -catenin, we next examined whether Wnt3a-CM enhances the formation of the β -catenin-AR protein complex in nuclei. Using the nuclear fraction of LNCaP cells treated with Wnt3a-CM, we assessed the levels of β -catenin in the protein complex with AR by coimmunoprecipitation. We observed more β -catenin proteins interacting with AR in cells treated with Wnt3a-CM than in cells treated with the control medium in the absence of 1 nmol/L DHT (Fig. 3C). As described previously, we also observed that β -catenin forms a protein complex with the AR in LNCaP cells in the presence of androgens. The data provide another line of evidence indicating that Wnt3a-CM induces the formation of AR- β -catenin protein complexes in the nucleus.

Wnt3a-CM Promotes Cell Growth and Colony Formation in the Absence of Androgens. Next, we investigated the role of Wnt3a-CM in the regulation of LNCaP cell growth. In particular, we addressed whether Wnt3a-CM can function as a growth factor to

promote LNCaP cell growth in a ligand-independent manner. LNCaP cells were cultured with Wnt3a-CM that was prepared in RPMI 1640 with 10% CS-FCS (see Materials and Methods). The growth of LNCaP cells was first assessed by the MTS assay. In the presence of Wnt3a-CM, the cell numbers were 20% and 35% higher after 4 and 6 days compared with controls (Fig. 4A). We then assessed the growth-promoting effect of Wnt3a-CM using a colony formation assay. Approximately 500 LNCaP cells were seeded in each well and incubated with Wnt3a-CM. After 12 days, cells were fixed and stained with crystal violet. There are more and larger colonies in the samples incubated with Wnt3a-CM than in the ones treated with control medium (Fig. 4B). The number of colonies containing >50 cells is significantly higher in the samples treated with Wnt3a-CM than in the controls ($P < 0.001$; Fig. 4C). The above-mentioned data demonstrate that Wnt3a-CM increases the growth of prostate cancer cells in the absence of androgens. We also performed the above experiments in the presence of 0.1 nmol/L DHT and observed a clear effect of Wnt3a-CM in enhancing the growth of prostate cancer cells (data not shown).

Purified Wnt3a Proteins Enhance AR-Mediated Transcription and Cell Growth. Recently, Wnt molecules, including the product of the mouse Wnt3a gene, have been isolated (29) and appear active in inducing self-renewal of hematopoietic stem cells. We found that, like Wnt3a-CM, purified Wnt3a proteins can enhance the level of cytosolic β -catenin in two prostate cancer cell lines, PC3 and LNCaP (Fig. 5A). Then, we tested the activity of purified Wnt3a proteins in transient transfection assays. The PSA-luc plasmids were transfected with or without a wild-type AR expression vector into PC3 cells, which are AR negative. In the presence of 0.1 nmol/L DHT, Wnt3a-CM induces approximately 35% to 40% of AR-mediated transcription (Fig. 5B). Intriguingly, under a similar experimental condition, purified Wnt3a proteins show a potent and dosage-dependent enhancement of AR transactivation. To confirm this finding, we repeated transient transfection experiments in LNCaP cells. As shown in Fig. 5C, purified Wnt3a proteins show a similar induction of AR-mediated transcription in the presence or absence of 0.1 nmol/L DHT. However, the effect is more pronounced in the cells treated with the ligand. To further assess the growth-promoting effect of purified Wnt3a proteins, we repeated the colony formation assays. We ob-

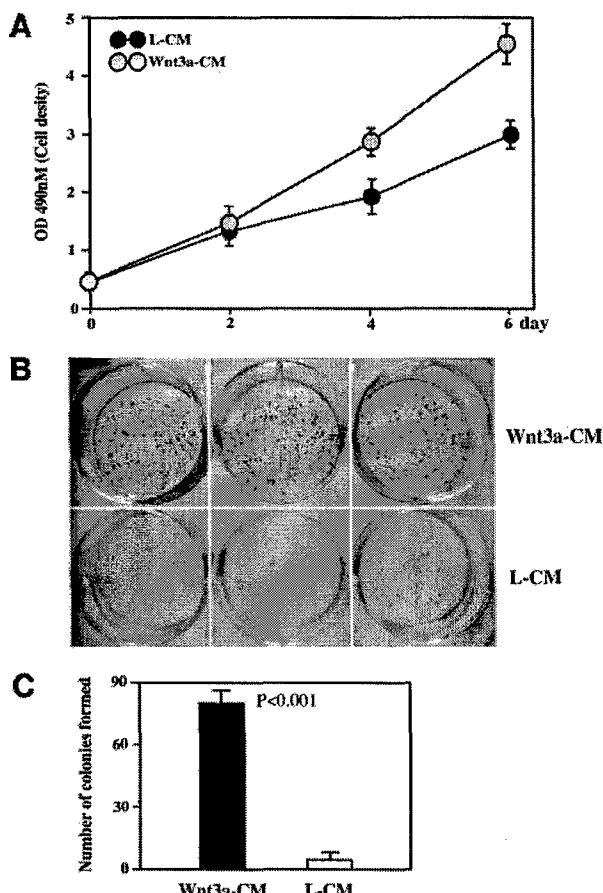


Fig. 4. Wnt3a-CM promotes the growth of LNCaP cells in a ligand-independent manner. *A*, LNCaP cells were cultured with Wnt3a-CM or L-CM in the absence of DHT. At the indicated time points, cells were harvested and analyzed by the MTS assay. The data represent the mean \pm SD of three independent experiments. *B*, For the colony formation assay, 500 LNCaP cells were seeded in 6-well plates and cultured in Wnt3a-CM or L-CM in the absence of DHT. Cells were fixed and stained with crystal violet after a 12-day incubation. *C*, Colonies containing >50 cells were counted and analyzed. The results are from three separate transfection experiments.

served an increase of colony size and number in the samples treated with purified Wnt3a proteins compared with the ones treated with buffer only (Fig. 5D). The number of colonies containing >50 cells is significantly higher in the samples treated with purified Wnt3a proteins than in the controls ($P < 0.001$; data not shown). Taken together, the above results confirm an important role of the Wnt3a proteins in AR-mediated transcription and prostate cell growth.

DISCUSSION

Wnt signaling pathways regulate a variety of processes including cell growth, development, and oncogenesis (13, 34). However, the biological roles of the Wnt growth factors serving as the upstream signaling of β -catenin have not been fully characterized in prostate cancer cells. In this study, we investigate whether there is a direct effect of the Wnt growth factor on AR-mediated transcription and its role in the growth of prostate cancer cells.

Wnt3a-CM prepared from mouse L cells stably transfected with mouse Wnt3a cDNA has been well characterized (24). It has become a great resource and is used frequently to study the Wnt signaling pathway (8, 35). Previous studies have shown that Wnt3a-CM increases the cytosolic and nuclear levels of β -catenin (8, 24). Microarray data demonstrated that treatment of human embryonic carcinoma with Wnt3a-CM up-regulated the expression of β -catenin, down-

stream target genes of TCF/LEF, and other factors involved in the regulation of β -catenin (35). These multiple lines of evidence confirm a specific signaling pathway mediated by Wnt3a-CM in cells.

In this study, we showed that Wnt3a-CM stimulates AR-mediated transcription. We demonstrated that Wnt3a-CM is capable of inducing AR-mediated transcription from the PSA promoter/reporter and the expression of endogenous PSA transcripts in a ligand-independent manner. In LNCaP cells, the stimulation by Wnt3a-CM of AR is very effective and is almost as great as the effect achieved by adding 1 nmol/L DHT. In addition, our data also showed that Wnt3a-CM is able to increase AR-mediated transcription in the presence of low concentrations of DHT. These data provide the first line of evidence showing a unique and important role of Wnt3a in the regulation of the androgen signaling pathway in a ligand-dependent manner.

To understand the molecular mechanism by which Wnt3a augments AR-mediated transcription, we performed several experiments to confirm the involvement of the AR in the regulation. We showed that Wnt3a-CM induces transcription of the 7-kb PSA promoter-luc and endogenous PSA gene. Moreover, Wnt3a-CM also affects a minimal promoter containing only two AREs. Furthermore, we demonstrated that induction of AR-dependent promoters by Wnt3a-CM can be completely abolished by an AR shRNA construct and an AR antagonist, bicalutamide. These data implicate that induction by Wnt3a-CM is mediated through the AR.

β -Catenin plays a central role in the Wnt signaling pathway. As reported previously, we observed an increase in free cytosolic and nuclear β -catenin in both prostate and nonprostate cancer cells that were treated with Wnt3a-CM (Fig. 1A and B). Previous studies by us and others have shown that β -catenin is a coactivator of AR (19–21). Therefore, we examined whether β -catenin is a downstream effector of Wnt3a, augmenting AR-mediated transcription. Using an antisense construct of β -catenin, we were able to partially block the effect of Wnt3a-CM on AR-mediated activity. Moreover, overexpression of TCF or E-cadherin, β -catenin-binding proteins, also reduced the Wnt3a-CM-mediated AR activity on the PSA promoter. These data suggest an involvement of β -catenin in Wnt3a-CM-induced AR transcription, which is in agreement with the previous finding that β -catenin acts as an AR coactivator (19–21). However, given the fact that expression of the antisense β -catenin and TCF and E-cadherin constructs only partially blocks the effect of Wnt3a-CM, it appears that other factors and/or pathways may also be involved in the regulation. It has been shown that Wnt growth factors/ligands can stimulate both canonical and non-canonical pathways. In this regard, the molecular mechanism(s) by which Wnt growth factors regulate the androgen signaling in prostate cells must be explored further.

Although the mechanisms by which prostate cancer cells develop into the androgen-insensitive stage are currently unclear, it is believed that the tumor cells must either bypass or adapt the androgen signaling pathway to survive in a low-androgen environment during progression. AR mutations have been identified in some androgen-insensitive prostate cancers (36–38). Amplification of the AR gene has been observed in some biopsy samples during androgen ablation therapy (39). Recent studies showed that the modulation of the AR protein by phosphorylation, acetylation, and sumoylation also regulates AR activity (40–43). In particular, it has been shown that phosphatidylinositol 3'-kinase/Akt and PTEN regulate AR-mediated transcription through either direct phosphorylation of AR proteins (44) or modification of AR cofactors, such as β -catenin (27). In the current study, we provide several lines of evidence demonstrating that Wnt3a is able to stimulate AR-mediated transcription in the absence of ligand or the presence of a low level of ligand in prostate cancer cells. The above-mentioned data suggest that signals delivered through the AR are still essential in androgen-insensitive prostate cancer cells. Intriguingly,

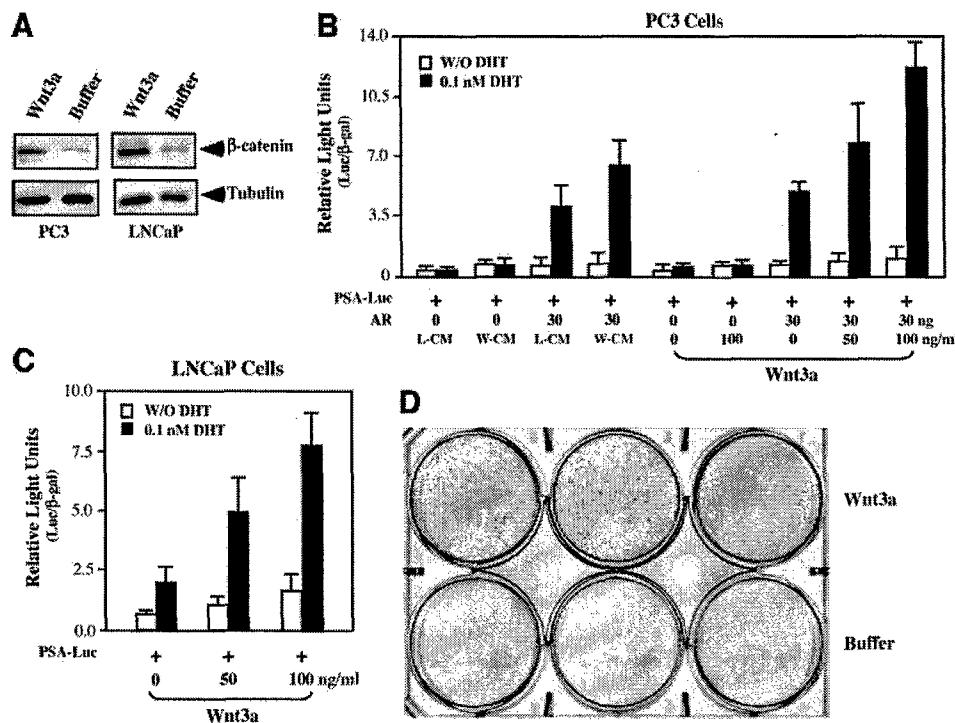


Fig. 5. Purified Wnt3a proteins enhance AR-mediated transcription and cell growth. *A*, Both PC3 and LNCaP cells were cultured in the presence of purified Wnt-3a (100 ng/mL) or the elution buffer (*Buffer*) as a control for 24 hours. Cytosolic fractions were isolated and analyzed by Western blot. *B*, PC3 cells were transiently transfected with 100 ng of PSA-Luc, 25 ng of pcDNA3- β -Gal, and 30 ng of pcDNA3-AR; washed after 24 hours; and then incubated in Wnt-3a-CM or L-CM or treated with the indicated amounts of purified Wnt-3a proteins in the presence or absence of 0.1 nmol/L DHT. Luciferase and β -Gal activities were measured as described previously. *C*, LNCaP cells were transiently transfected with 100 ng of PSA-Luc and 25 ng of pCMV- β -Gal. After 24 hours, transfected cells were washed and incubated with the indicated amounts of purified Wnt-3a. *D*, Approximately 1,000 LNCaP cells were plated in triplicate in DMEM containing 10% CS-FCS in the presence of 100 ng/mL Wnt-3a. Ten days after incubation, the cells were stained with crystal violet. A representative plate is shown. Colonies containing >50 cells were counted and analyzed as described in Fig. 4C.

we also show that Wnt3a-CM is able to promote the growth of prostate cancer cells in a ligand-independent manner. The fact that Wnt3a-CM can promote cell growth and induce AR-mediated transcription suggests a unique role of the Wnt growth factor in the progression of prostate cancer cells from the androgen-sensitive to -insensitive stages. It is possible that aberrant expression of Wnt growth factors and/or their receptors in prostate cancer tissues may play a critical role in the progression of prostate cancer.

Although attempts to purify Wnt proteins have been hampered by several technical difficulties, including their high degree of insolubility, active Wnt molecules, including the product of the mouse Wnt3a gene, have been isolated recently (29). In this study, we first showed that, like Wnt3a-CM, purified Wnt3a proteins increase the cytosolic level of β -catenin in prostate cancer cells. Then we confirmed the role of purified Wnt3a proteins on AR-mediated transcription and cell growth in prostate cancer cells. In PC3 cells, Wnt3a proteins enhance AR-mediated transcription in a dosage-dependent manner. A similar effect by purified Wnt3a proteins was also observed in LNCaP cells. These data provide a direct line of evidence demonstrating a true effect of Wnt3a on AR-mediated transcription in prostate cells. However, we observed that purified Wnt3a proteins only slightly affect AR activity in the absence of DHT. This is different from the results that we observed in the experiments when Wnt3a-CM was used. Currently, we do not know the exact reason(s) why purified Wnt3a proteins have less effect on AR-mediated transcription than Wnt3a-CM in the absence of androgens. Further characterization of different protein fractions during purification processes may lead to the identification of additional factors or cofactors that contribute to Wnt3a-mediated augmentation of AR activity. Additional studies of the expression profiles of Wnt ligands and receptors in prostate tissues and prostate cancer cells will also help us to fully understand the signaling pathway(s) regulated by Wnt growth factors in prostate cancer cells.

In this study, we provide several lines of evidence that Wnt3a acts as an upstream signal to induce the transcriptional activity of AR and the growth of prostate cancer cells, possibly through β -catenin. In

particular, using purified Wnt3a proteins, we confirm the important role of the Wnt3a growth factor in inducing AR-mediated transcription and cell growth. The effect of Wnt3a may play a critical role in maintaining or increasing AR activity in the setting of decreased androgen levels during androgen ablation therapy. Therefore, further study of the molecular mechanisms by which Wnt growth factors modulate androgen signaling should provide fresh insight into the progression of prostate cancer, which may help us to identify new steps that can be targeted for prostate cancer treatment.

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β -Catenin Is Involved in Insulin-Like Growth Factor 1-Mediated Transactivation of the Androgen Receptor

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The androgen-signaling pathway is important for the growth and progression of prostate cancer cells. IGF-I and other polypeptide growth factors have been shown to be capable of induction of androgen receptor (AR) activation in the absence of, or at low levels of, ligand. It has been shown that IGF-I increases the cellular level of β -catenin, an AR coactivator. In this study, we performed several experiments to test whether β -catenin is involved in IGF-I-induced AR-mediated transcription. We demonstrate that IGF-I enhances the expression of endogenous prostate-specific antigen, an AR target gene, and elevates the level of

cytoplasmic and nuclear β -catenin in prostate cancer cells. Transfection of either wild-type or a constitutively active mutant of the IGF-I receptor augments AR-mediated transcription. An anti-sense construct of β -catenin that decreases the cellular level of β -catenin can reduce IGF-1 receptor-mediated enhancement of AR activity. Moreover, using a pulse-chase experiment, we showed that IGF-I enhances the stability of β -catenin in prostate cancer cells. Our findings delineate a novel pathway for IGF-I in modulating androgen signaling through β -catenin. (Molecular Endocrinology 19: 391-398, 2005)

THE FACT THAT androgen ablation is an effective treatment for the majority of metastatic prostate cancer patients indicates that androgen plays an essential role in regulating the growth of prostate cancer cells (1). Unfortunately, most patients develop androgen-insensitive prostate cancer within 2 yr, for which there is currently no effective treatment. Failure of androgen ablation suggests that there are alterations in androgen signaling in the tumor cells. Multiple mechanisms by which prostate cancer cells progress to androgen-insensitive stages have been proposed (2).

Previous experiments have shown that androgen receptor (AR) can be activated in cells treated with polypeptide growth factors in the absence of, or at low levels of androgens (3). IGF-I is the most efficient growth factor capable of ligand-independent activation of AR. However, the mechanisms by which IGF-I or other growth factors regulates AR-mediated transcription in prostate cells remain unclear. Recently, Playford *et al.* (4) reported that IGF-I enhances tyrosine phosphorylation of β -catenin in human colorectal can-

cer cells, which results in dissociation of β -catenin from E-cadherin complexes at the cell membrane. Induction by IGF-I also increases the stability of the β -catenin protein (4). These results suggest a new link between β -catenin and IGF-I-mediated cell growth and transformation.

Recently, we and others (5-8) have demonstrated a specific protein-protein interaction between β -catenin and AR. Through the interaction, β -catenin augments AR-mediated transcription. β -Catenin plays a pivotal role in cadherin-based cell adhesion and in the Wnt signaling pathway [see the review by Polakis (9)]. Most β -catenin is located in the cell membrane where it is associated with the cytoplasmic region of E-cadherin, a transmembrane protein involved in homotypic cell-cell contacts (10). Smaller pools of β -catenin are located in both the nucleus and cytoplasm, where the protein mediates Wnt signaling. The Wnt signal and E-cadherin can modulate the cellular level of β -catenin (6, 11). Accumulated β -catenin translocates into the nucleus and forms transcriptionally active complexes with Tcf/LEF (12) or the AR (5, 6). AR can modulate Tcf/LEF-mediated cellular effects by binding to limiting amounts of β -catenin, which may be critical during normal prostate development and tumor progression (13, 14).

Based on the facts that β -catenin is an AR coactivator and that IGF-I affects AR-mediated transcription, we investigated the molecular mechanism by which IGF-I enhances AR activity. Particularly, we addressed whether the activation of AR by IGF-I in prostate cells is modulated by the cellular level of β -catenin. In the human prostate cancer cell line, LNCaP, we show that

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Abbreviations: AR, Androgen receptor; ARE, androgen response element; DHT, dihydrotestosterone; FCS, fetal calf serum; β -gal, β -galactosidase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK3 β , glycogen synthase kinase-3 β ; H1, histone; IGF-1R, IGF-I receptor; PI3K, phosphoinositol 3-kinase; PSA, prostate-specific antigen.

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IGF-1 enhances AR-mediated transcription at a low level of androgen, and that it increases the level of cellular β -catenin. Transfection of either wild-type or a constitutively active mutant of the IGF-1 receptor (IGF-1R) augments AR-mediated transcription. Moreover, using an antisense construct of β -catenin, we further demonstrate that a decrease in the cellular level of β -catenin can reduce IGF-1R-mediated enhancement of AR activity. Furthermore, pulse-chase experiments demonstrate that IGF-1 enhances the stability of the β -catenin protein in prostate cancer cells. These findings delineate a novel mechanism by which IGF-1 modulates androgen signaling in prostate cells and provides fresh insight into the role of IGF-1 in the development of androgen-insensitive prostate cancer.

RESULTS

IGF-1 Enhances the Expression of Prostate-Specific Antigen (PSA), a Target Gene of AR, in Prostate Cells

Previous experiments showed that AR can be activated in cells treated with growth factors in the absence of, or at low levels of ligand (3). IGF-1 is the most efficient growth factor capable of ligand-independent activation of AR. To evaluate the effect of IGF-1 in a biologically relevant setting, we tested whether IGF-1 regulates expression of the PSA gene, an endogenous AR target, in the AR-positive prostate cancer cell line, LNCaP. Using real-time PCR, we first measured transcripts of PSA in LNCaP cells treated with different amounts of IGF-1. In the presence of 0.1 nM dihydrotestosterone (DHT), PSA expression was increased approximately 20 or 35% in LNCaP cells treated with 30 or 100 ng/ml of IGF-1, respectively, over that found in cells not treated with IGF-1 ($P < 0.05$) (Fig. 1A). However, in the absence of DHT, the expression of PSA was not significantly affected by IGF-1. To confirm this finding, we examined the expression of PSA by conventional Northern blotting. As observed in the real-time PCR assays, an increase in PSA transcripts was found in the cells treated with 30 or 100 ng/ml of IGF-1 (Fig. 1B). Using β -actin as a reference gene, we showed an approximately 0.3- to 1-fold increase in PSA transcripts in the cells treated with 30 or 100 ng/ml of IGF-1, respectively (Fig. 1C). These results provide the first line of evidence that IGF-1 is able to enhance endogenous AR-mediated transcription in prostate cancer cells in the presence of a low level of androgens.

IGF-1 Increases the Level of Cellular β -Catenin in Prostate Cancer Cells

It has been shown that IGF-1 can elevate the cellular level of β -catenin in the human colon cancer cell line, C10 (4). β -Catenin has been demonstrated to be an AR coactivator (5, 6). To determine whether β -catenin

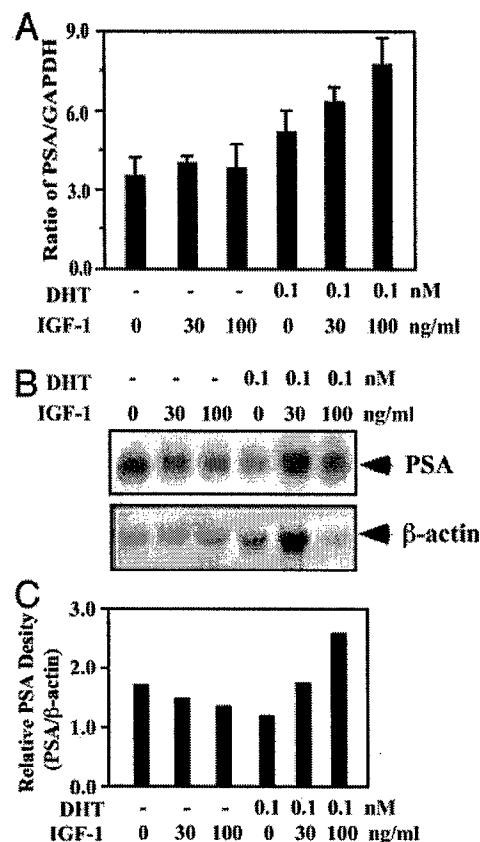


Fig. 1. IGF-1 Enhances the Transcription of an Endogenous AR Target Gene, PSA

A, Expression levels of PSA mRNA were quantified using quantitative fluorescent real-time PCR. Total RNAs were isolated from LNCaP cells cultured in T-medium with or without 0.1 nM DHT, treated for 16 h with IGF-1 or vehicle. RNA samples were first reverse-transcribed using random hexamers. Two specific primers selected from the regions around the translation initiation site or the stop codon of the PSA gene were used for amplification. PCR assays were performed with TaqMan PCR reagent Kits in the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The levels of PSA mRNA were normalized by coamplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA as described by the manufacturer (PE Applied Biosystems). B, Expression of the endogenous PSA transcripts was detected by a cDNA probe derived from the human PSA gene in LNCaP cells treated with IGF-1 as described above. A β -actin probe was used to confirm equal RNA loading. C, Densitometry of the Northern blot was performed, and the relative numbers are reported as PSA/ β -actin.

is involved in IGF-1 mediated AR transcription, we examined free cellular β -catenin in prostate cancer cells as described previously (4). As shown in Fig. 2A, there was no significant change in the amount of β -catenin in the cytoskeletal compartment (RIPA) of cells treated with IGF-1. However, there was a 2- to 4-fold increase in cytosolic β -catenin in cells treated with 50 or 100 ng of IGF-1 (Digi), respectively. In contrast, the level of cytosolic tubulin, used as a

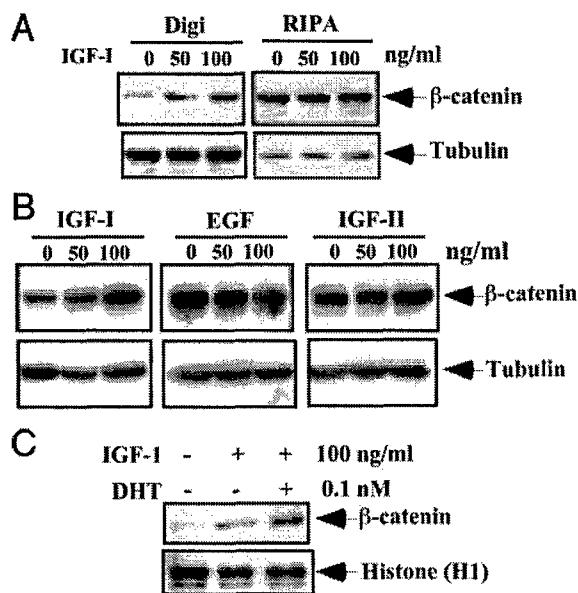


Fig. 2. IGF-I Signaling Enhances the Level of Free Cytosolic β -Catenin

A, Both cytosolic (Digi) and cytoskeletal fractions (RIPA) were prepared from LNCaP cells as described in *Materials and Methods*. Both β -catenin and tubulin proteins were analyzed by Western blotting with specific antibodies. B, Cytosolic fractions were isolated from LNCaP cells treated with different concentrations of growth factors. The levels of β -catenin were analyzed by Western blotting. Tubulin was used as a control for protein loading. C, The endogenous β -catenin proteins were analyzed in nuclear extracts isolated from LNCaP cells treated with or without IGF-I or DHT as labeled in the figure. The level of β -catenin was detected by Western blotting with a specific β -catenin antibody. H1, a nuclear protein, was used as a control for protein loading.

control, showed no significant difference in the treated and untreated cells.

To further test whether the effect of IGF-I on β -catenin is a specific event, we repeated the above experiments with LNCaP cells treated with different growth factors. As shown in the figure, a pronounced increase in cytosolic β -catenin was observed in cells treated with IGF-I but not with EGF (Fig. 2B). With 100 ng/ml of IGF-II, a slight change of β -catenin was also observed, suggesting a potential role of IGF-II in the regulation of cellular β -catenin (see *Discussion*). In addition, we also performed transient transfection experiments using the PSA-promoter/reporter in LNCaP cells in the presence of the above growth factors. We observed that IGF-I is one of the most efficient growth factors in modulating AR-mediated transcription (data not shown), which is consistent with the previous report by Culig *et al.* (15).

Next, we examined whether IGF-I directly affects the translocation of β -catenin to the nucleus. In the presence of 0.1 nm of DHT, IGF-I significantly increases the level of nuclear β -catenin in LNCaP cells, whereas it only affects β -catenin slightly in the absence of ligand (Fig. 2C). These data are consis-

tent with our previous observation that IGF-I has a more pronounced effect in enhancing AR-mediated transcription in the presence of low level of androgens. The histone (H1) protein, used as a control, showed no change (Fig. 2C). Taken together, these results confirm the role of IGF-I in enhancing the translocation of β -catenin into the nucleus, which agree with the previous studies showing the similar effect of IGF-I in enhancing nuclear β -catenin in human colorectal cancer cells (4).

Overexpression of IGF-1R Enhances AR-Mediated Transcription

To confirm that IGF-I enhances AR activity, we tested the ability of either the wild-type IGF-1R or a constitutively activated receptor, IGF-1R-NM1, generated by deleting the entire extracellular domain of IGF-1R and fusing the remaining receptor (16), to enhance AR-mediated transcription. In cells cultured with 5% fetal calf serum, both the wild-type IGF-I and IGF-1R-NM1 augmented AR-mediated transcription from the PSA promoter in a dose-dependent manner ($P < 0.05$) (Fig. 3A). The IGF-1R-NM1 receptor showed a stronger augmentation of AR-mediated transcription than the wild-type receptor both in the absence and presence of androgens ($P < 0.05$). These results provide an additional line of evidence that IGF-I signaling plays a role in regulating AR-mediated transcription.

To further demonstrate that the enhancement of AR activity by IGF-I receptors was mediated by β -catenin, we repeated the above experiments with an antisense construct of β -catenin (6). As shown in Fig. 3B, cotransfection of the antisense β -catenin plasmid represses the enhancement of AR by both the wild-type and the mutant IGF-1R. With 30 ng of the antisense construct, AR activity was reduced 45% and 65% in cells transfected with the wild-type and mutant IGF-1R ($P < 0.05$), respectively. To evaluate the effectiveness of β -catenin antisense constructs, we also measured the level of the cytosolic β -catenin protein in the above samples. As expected, the antisense β -catenin constructs reduced the levels of cytosolic β -catenin (Fig. 3C), which correlated with the reduction in AR transcriptional activity in the cells. To further demonstrate the role of β -catenin in IGF-I-induced AR activity, we examined whether the inhibition of β -catenin expression can affect IGF-I-induced AR activity by using the β -catenin antisense constructs. As shown in Fig. 3D, the IGF-I-induced AR transactivation of the PSA promoter is abrogated by cotransfection with the β -catenin antisense constructs. In addition, we also demonstrate that the specific IGF-1R antibody, α IR3, effectively blocks the IGF-I mediated AR activity. Taken together, the above results demonstrate a direct involvement of β -catenin in the IGF-I signaling modulated, AR-mediated transcription.

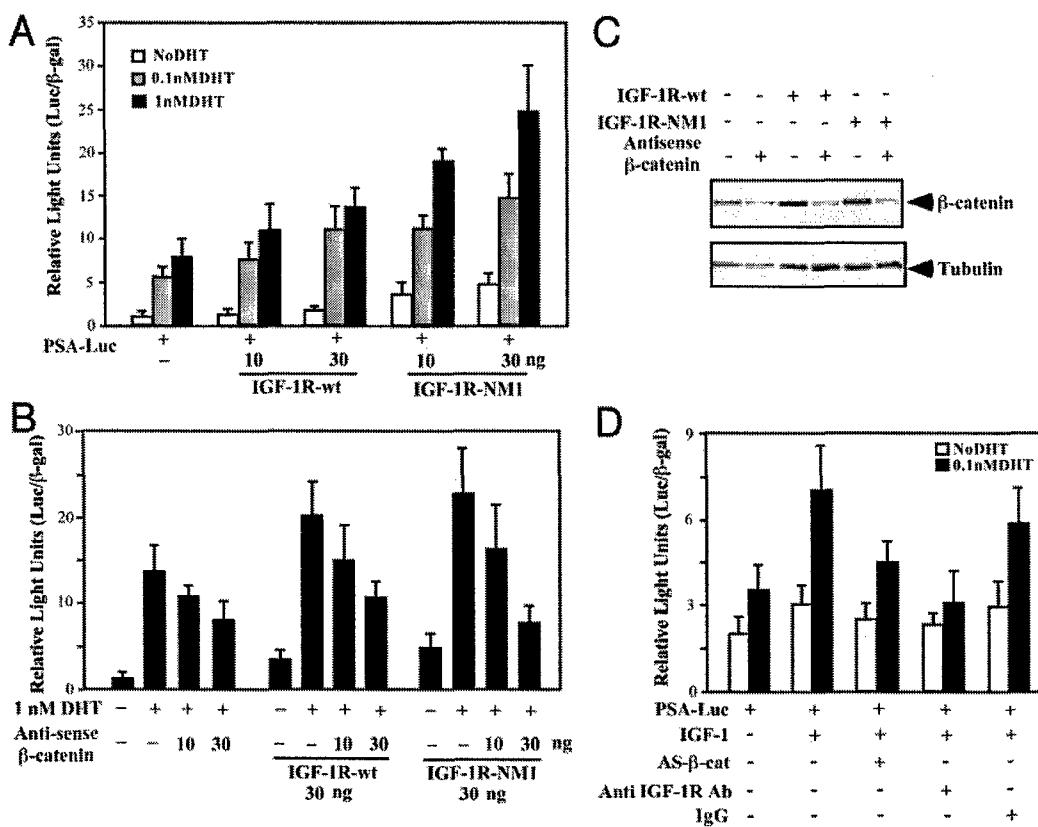


Fig. 3. Overexpression of IGF-I Receptors Enhances AR-Mediated Transcription

A, Transient transfections were performed in LNCaP cells with 100 ng of *PSA7kb-luc* reporter and 10 or 30 ng of wild type (wt) or a constitutively active mutant (NM1) of IGF-I receptor and 25 ng of pcDNA3-β-gal. The cells were incubated in T-medium with 5% charcoal-stripped FCS for 12 h, and then were treated with different concentrations of DHT for 18 h. Cell lysates were measured for luciferase and β-gal activities. The data represent the mean ± SD of three independent samples. B, The transfection experiments were repeated in LNCaP cells under the same conditions as described above. Different amounts of the antisense β-catenin plasmid were cotransfected into the cells. The relative light units (RLU) from individual transfections were normalized by measurement of β-gal activity expressed from a cotransfected plasmid in the same samples. C, The levels of cytosolic β-catenin were measured by Western blotting from LNCaP transfected with 30 ng of the IGF-I receptors and 30 ng of antisense β-catenin plasmids (AS-β-cat). D, One hundred nanograms of *PSA7kb-luc* reporter with or without 30 ng of the β-catenin antisense plasmids were transfected into LNCaP cells. The cells were cultured in T-medium with or without 100 ng/ml of IGF-I in the presence or absence of DHT for 28–32 h. IGF-1R antibody (1 μg/ml) (catalog no. GR11, CalBiochem, San Diego, CA) or mouse normal IgG was added into cells 6 h after transfection. Luciferase and β-gal activities were measured as described above.

IGF-I Stabilizes β-Catenin in Prostate Cancer Cells

It has been shown that IGF-I enhances the stability of the β-catenin protein in human colorectal cancer cells (4). IGF-I inhibits glycogen synthase kinase-3β (GSK3β) by stimulating the phosphorylation of GSK3β (17, 18). We therefore examined whether the IGF-I enhancement of the stability of β-catenin in prostate cancer cells is mediated through GSK3β. Because LiCl has been shown to repress GSK3β (19), thereby stabilizing cellular β-catenin, we measured the cytosolic level of β-catenin in LNCaP cells treated with or without IGF-I. As observed previously, we saw an increase in β-catenin in cells treated with IGF-I (Fig. 4A). In the presence of 50 mM LiCl, the samples isolated from cells treated with

100 ng/ml of IGF-I showed higher levels of β-catenin than ones not treated with LiCl. We conclude that inhibition of GSK3β can further enhance the levels of cellular β-catenin induced by IGF-I in prostate cancer cells.

To demonstrate that the effect of IGF-I was on the stability of β-catenin in prostate cancer cells, we performed pulse-chase experiments in LNCaP cells. Cells were pulsed with 35 S label and chased in the presence or absence of IGF-I for 12 h. 35 S-labeled β-catenin proteins were immunoprecipitated from the cytosolic fractions and analyzed by SDS-PAGE. As shown in Fig. 4B, IGF-I enhances the stability of β-catenin, increasing its half-life from 9–12 h. These results provide a direct line of evidence that IGF-I indeed affects the stability of β-catenin in prostate cancer cells.

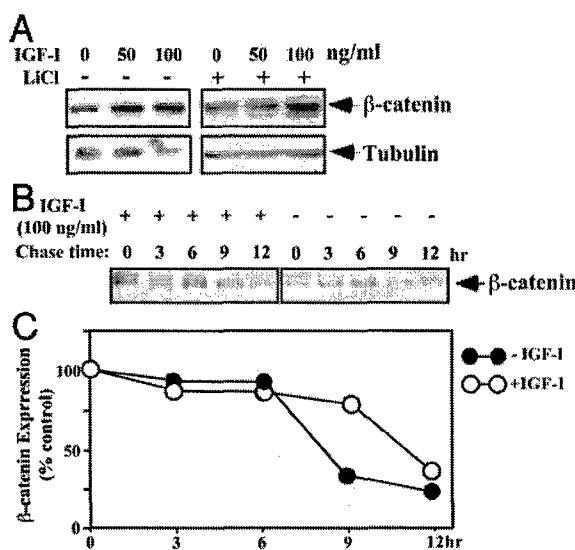


Fig. 4. IGF-I Enhances the Stability of β -Catenin

A, The cytosolic fraction was used to measure the free form of β -catenin in LNCaP cells treated with different concentration of IGF-I in the presence or absence of 50 mM LiCl. B, LNCaP cells were pulsed with Tran^{35}S -label and chased with medium containing an excess of cold methionine/cysteine for the indicated times. The cytosolic fractions isolated from cells were immunoprecipitated for β -catenin. C, The results were analyzed by densitometry and expressed graphically as a percentage of the value at 0 h. The figure shows results of a single experiment, which was repeated once with similar results.

DISCUSSION

Induction of ligand-independent activation of AR by IGF-I and other polypeptide growth factors was initially observed several years ago (3). However, the mechanism(s) by which the growth factors induce AR-mediated transcription were still unknown. It has been shown that IGF-I increases the cellular level of β -catenin in human colon cancer cells (4). In addition, β -catenin has been demonstrated to interact with AR and to enhance AR-mediated transcription (5, 6, 8, 20). The results described here provide multiple lines of evidence demonstrating that β -catenin is involved in the induction by IGF-I of AR-mediated transcription. This was demonstrated by experiments showing that IGF-I enhanced expression of PSA in prostate cells, that IGF-I increased the levels of β -catenin at least in part by stabilizing the protein, and finally, that increasing the levels of IGF-1R in prostate cells enhances the level of AR-mediated transcription.

We showed that IGF-I enhances the expression of endogenous PSA transcripts in cells treated with low levels of the DHT by both real-time PCR and Northern blot experiments. These results are different from those of Culig *et al.* (3), who showed previously that induction of AR activity by IGF-I on reporters driven by the androgen response element (ARE)- or murine mammary tumor virus promoters

was an androgen-independent effect. Obviously, there are several differences between our and their experiments. Although we do not know the exact reason(s) for these differences, we feel that our experiments, which examine the PSA transcripts, a natural, endogenous AR-target gene, are more sensitive and biologically relevant for assessing AR activity. It has been shown that the unbound AR forms a complex with heat-shock proteins (21), and upon binding to ligand, the AR dissociates from the heat-shock proteins and translocates into the nucleus (22). Our data agree with the previous observations, suggesting that the IGF-I-induced AR activity requires the nuclear translocation of AR upon binding to ligand, which is also supported by the recent observation that β -catenin augmenting the AR-mediated activity is a nuclear effect and requires the nuclear translocation of AR in the presence of the ligand (6, 8).

LNCaP is currently the only well-characterized prostate cancer cell line that contains both functional AR and E-cadherin pathways and is responsive to IGF-I (23–25). However, it has been shown that there are a relatively low number of IGF-I receptors on these cells, approximately 1×10^4 receptors/cell (25). To examine the effect of having a more physiologically relevant number of receptors on the cells, we transfected wild-type IGF-1R and a constitutively activated mutant of IGF-1R into LNCaP cells. Both the wild-type and the mutant IGF-1R enhanced AR activity from the 7-kb PSA promoter in a dose-dependent manner, indicating that IGF signaling modulates AR-mediated transcription. We also performed transient transfection experiments with a luciferase reporter driven by a minimal promoter with two AREs in LNCaP cells. We only observed a moderate effect of IGF-1R on the ARE-reporter, in contrast to the PSA-reporter (data not shown). This result may suggest that other transcription factors that bind to sites adjacent to the ARE in the natural PSA-promoter may enhance the AR in response to IGF-I-mediated induction.

The cellular levels of β -catenin are tightly regulated in normal cells. Mutations affecting the degradation of β -catenin can result in the accumulation of the cellular β -catenin to induce neoplastic transformation (26). Due to an abnormal cadherin-catenin interaction in the cell membrane, increasing the cytoplasmic and nuclear levels of β -catenin as a consequence of loss of E-cadherin is also frequently observed in late stages of prostate cancer cells (27). In this current study, intriguingly, we demonstrated that IGF-I increases the cellular levels of β -catenin in the prostate cancer cell line, LNCaP, which suggests a novel mechanism by which IGF-I modulates AR mediated transcription. Using both Western blot and pulse-chase assays, we further showed that an increase in β -catenin levels by IGF-I in LNCaP cells is the result of stabilization of β -catenin. Previous studies have shown that GSK3 β is one of the major components in the destruction complex that constitutively down-regulates the level of cellular

β -catenin. The signaling mediated by phosphoinositol 3-kinase (PI3K)/Akt can regulate GSK3 β through the phosphorylation of the protein (28). The tumor suppressor, phosphatase and tensin homolog deleted on chromosome 10 (PTEN), negatively regulates the PI3K pathway by blocking activation of Akt (29). Our observation that lithium chloride, an inhibitor of GSK3 β , enhances the stabilizing effect of IGF-I on β -catenin suggesting a potential link between the IGF-I and PI3K/Akt signaling pathways in modulating Wnt/ β -catenin signaling. Our results are also consistent with an earlier report that showed that IGF-I can stabilize the β -catenin protein in combination with lithium chloride in a human colon cancer cell line (4). Further studies of the interaction between IGF, PI3K/Akt, and Wnt/ β -catenin pathways may help us to understand their roles in cell-cell adhesion, cell migration, transformation, and tumor metastasis.

In this study, we showed that IGF-I enhances AR-mediated transcription and increases the levels of cellular β -catenin. In addition, we also observed a slight increase in β -catenin in cells treated with 100 ng/ml IGF-II (see Fig. 2B). This result suggests a possible role for IGF-II in the regulation of cellular β -catenin, which is consistent with the recent report that showed that IGF-II induces rapid β -catenin relocation to the nucleus during epithelium to mesenchyme transition (30). To further evaluate the roles of IGF-II and EGF in AR-mediated transcription, we also performed transient transfection experiments using the PSA-promoter/reporter in LNCaP cells. We observed a notable induction only by IGF-I in our experiments (data not shown), which is similar to the previous report by Culig *et al.* (3).

In conclusion, in this study we provide several lines of evidence linking IGF-I to the regulation of β -catenin. Because β -catenin has been identified as an AR co-regulator, demonstration of a link between IGF-I and β -catenin suggests a potential, novel mechanism by which IGF-I regulates prostate cancer cells in their progression to the androgen-insensitive stage. Further study of this linkage may help us to understand the roles of IGF-I signaling in prostate cancer pathogenesis.

MATERIALS AND METHODS

Real-Time PCR and Northern Blot Analysis

Total RNAs were isolated from LNCaP cells treated with IGF-I in the presence or absence of 0.1 nM DHT by using an RNAwiz kit (Ambion, Austin, TX), and RNA concentration was estimated from absorbance at 260 nm. Expression levels of PSA mRNA were quantified using quantitative fluorescent real-time PCR. RNA was first reverse-transcribed using random hexamers as described by the manufacturer (PE Applied Biosystems, Foster City, CA). Two specific primers selected from the regions around the translation initiation site or the stop codon of the PSA gene were used for amplification. PCR assays were performed with TaqMan PCR reagent Kits in the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The levels of PSA mRNA were normalized by coamplification of GAPDH mRNA as described by the man-

ufacturer (PE Applied Biosystems). Northern blotting assays were performed as described previously (31).

Cell Cultures and Transfections

The monkey kidney cell line, CV-1, was maintained in DMEM supplemented with 5% fetal calf serum (FCS) (HyClone, Denver, CO). An AR-positive prostate cancer cell line, LNCaP, was maintained in T-medium (Invitrogen Life Technologies, Carlsbad, CA) with 10% FCS. Transient transfections were carried out using LipofectAMINE 2000 (Invitrogen) as described previously (31). In the experiments with IGF-I and other growth factors (Sigma, St. Louis, MO), cells were usually cultured in T-medium for 16 h, and then were treated with different concentrations of growth factors for 20–24 h. For androgen induction experiments, cells were grown in T-medium with charcoal-stripped fetal calf serum (HyClone) for 16–24 h in the presence or absence of DHT.

Whole Cell and Nuclear Extracts

Both whole cell lysate and nuclear extracts were prepared as described previously (6, 32). The cytosolic or cytoskeletal fractions were prepared in digitonin lysis buffer [1% digitonin, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂] or in RIPA buffer [0.5% Nonidet P-40, 0.3% Triton X-100, 15 mM MgCl₂, 5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl (pH 7.8)], respectively (4). Protein fractions for immunoblotting were boiled in sodium dodecyl sulfate-sample buffer and then resolved on a 10% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and probed with anti- β -catenin antibody (catalog no. C19220, Transduction Labs, Lexington, KY). Proteins were detected using the ECL kit (Amersham, Arlington Heights, IL). The antibody against H1 (Santa Cruz Biotechnology, Santa Cruz, CA) or tubulin (Neomarker, Fremont, CA) was used for protein loading.

Plasmids

The reporter plasmid, pPSA7kb-luc, was provided by Dr. Jan Trapman (33). IGF-1R expression vectors were the kind gift of Dr. Weiqun Li (16). The antisense construct of human β -catenin and the pcDNA3-FLAG- β -catenin vector were generated as described previously (6).

Luciferase and β -Galactosidase (β -gal) Assay

Luciferase and β -gal activities were measured as previously described (6, 32). The relative light units from individual transfections were normalized by measurement of β -gal activity expressed from a cotransfected plasmid in the same samples. Individual transfection experiments were done in triplicate and the results are reported from representative experiments.

Pulse-Chase

LNCaP cells were transfected with wild-type pcDNA3-FLAG- β -catenin. After 24 h of transfection, the cells were incubated with DMEM without L-methionine and L-cysteine (Invitrogen Life Technologies) for 1 h, and then pulse-labeled with 100 μ Ci of Tran ³⁵S Label (ICN, Irvine, CA) for 30 min. The cells were washed twice with PBS and then chased by incubating in complete DMEM in the presence or absence of IGF-I (50 ng/ml) for various periods of time. The cells were lysed in RIPA and digitonin lysis buffers containing protease inhibitors. ³⁵S-labeled β -catenin protein was immunoprecipitated from the cytosolic fractions using an anti- β -catenin rabbit polyclonal antibody (H-102; Santa Cruz Biotechnology) and analyzed by SDS-PAGE.

Statistical Analysis

The difference in the values between two groups was analyzed using the Student's *t* test. $P < 0.05$ was considered statistically significant.

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